

THE REACTIONS OF PROTEINS
WITH THIOLS AND DISULPHIDES

A THESIS

presented for the degree of

Doctor of Philosophy

in The University of Edinburgh

by

T. E. ISLES, B.Sc.



CONTENTS

| | <u>Page</u> |
|---|-------------|
| <u>INTRODUCTION</u> | |
| The Functions of Sulphydryl and Disulphide groups. | 3 |
| Types of Sulphydryl Group. | 5 |
| The Sulphydryl-Containing Proteins Studied. | 6 |
| Non-Protein Thiols and Disulphides. | 12 |
| Thiol - Disulphide Interactions. | 14 |
| <u>METHODS</u> | |
| General Introduction. | 24 |
| Preparation of Proteins. | 28 |
| Estimation of Protein Concentrations. | 31 |
| Purification of p-Chloromercuribenzoate. | 31 |
| A Method for the Estimation of Protein Sulphydryl Content. | 33 |
| The Estimation of Non-Protein Sulphydryl Compounds. | 50 |
| (a) The pCMB method | 50 |
| (b) The Alloxan '305' method | 52 |
| The Estimation of Non-Protein Disulphides. | 56 |
| (a) The estimation of GSSG | 57 |
| (b) The estimation of CySSCy | 61 |
| <u>ACKNOWLEDGMENTS</u> | |
| The Removal of Unreacted GSH and GSSG from Reaction Mixtures. | 64 |

| | <u>Page</u> |
|--|-------------|
| <u>RESULTS</u> | |
| Studies with the Haemoglobins. | 66 |
| The following abbreviations were used widely in the | |
| 1. Human Haemoglobin | 67 |
| 2. Bovine Haemoglobin | 70 |
| (a) Haemoglobin and GSSG | 70 |
| (b) Haemoglobin and GSH | 70 |
| (c) Haemoglobin and CySSCy | 74 |
| Bovine Gamma Globulin and GSSG. | 77 |
| Reaction of Serum Albumin with Thiols and Disulphides. | 79 |
| 1. Bovine Serum Albumin and GSSG | 79 |
| 2. Human Serum Albumin and GSSG. | 85 |
| 3. Reaction of Bovine Serum Albumin with CySSCy | 87 |
| (a) Effect of pCMB | 92 |
| (b) Rate of the reaction | 95 |
| (c) Factors influencing the reaction | 95 |
| (d) Kinetics of the reaction | 102 |
| (e) Reversal of the reaction | 105 |
| 4. Reaction of Bovine Serum Albumin with other thiols and disulphides. | 116 |
| Reaction of CySSCy with Human Plasma. | 119 |
| <u>DISCUSSION</u> | 126 |
| <u>SUMMARY</u> | 142 |
| <u>ACKNOWLEDGMENTS</u> | 145 |
| <u>REFERENCES</u> | 146 |

ABBREVIATIONS

The following abbreviations were used widely in the thesis.

| | |
|--------------------|---|
| GSH | reduced glutathione |
| GSSG | oxidised glutathione |
| CySH | cysteine |
| CySSCy | cystine |
| HSA | Human serum albumin |
| BSA | Bovine serum albumin |
| pCMB | sodium p-chloromercuribenzoate |
| NADPH ₂ | reduced nicotinamide-adenine- dinucleotide phosphate |
| NADH ₂ | reduced nicotinamide-adenine dinucleotide |

INTRODUCTION

An extensive study of the chemistry of sulfur
has been made and many important results have been
obtained in the last few years.

Many of the results of this study have been
published in the last few years, and many of the
results of this study have been published in the last
few years.

The study of the chemistry of sulfur has been
extensive and many important results have been
obtained in the last few years.

INTRODUCTION

The study of the chemistry of sulfur has been
extensive and many important results have been
obtained in the last few years.

The study of the chemistry of sulfur has been
extensive and many important results have been
obtained in the last few years.

The study of the chemistry of sulfur has been
extensive and many important results have been
obtained in the last few years.

The study of the chemistry of sulfur has been
extensive and many important results have been
obtained in the last few years.

INTRODUCTION

An advance in our knowledge of the chemistry of sulphur compounds occurring in biological systems has been seen over the last few years.

Much has been learned of the functions of sulphhydryl compounds in such systems, while studies on protein disulphide groups have suggested that their main role lies in the maintenance of the specific structure of the proteins.

The sulphhydryl group has undoubtedly received more attention than any other reactive group in protein chemistry, not only because there are so many, more or less specific, reagents available for its detection, but also because the presence of sulphhydryl groups has been found to be essential for a number of enzymic reactions.

Non-protein sulphhydryl compounds have only recently been systematically studied and the knowledge which has been acquired about their function, distribution and fate is for the most part fragmentary and somewhat disconnected.

The interaction of proteins, containing disulphide and/or sulphhydryl groups, with non-protein thiols and disulphides although recognised for over thirty years was not studied so systematically, or so successfully, until the advent of ^{35}S -labelled sulphur compounds and suitable analytical techniques /

techniques in the late nineteen forties.

It is difficult to establish a date for the discovery of sulphydryl and disulphide compounds as natural constituents of biological systems.

Using the nitroprusside reaction, introduced by Morner (1899) for the identification of Cysteine, Heffter (1907) and Arnold (1910) obtained positive tests with extracts of tissues; with plasma proteins, treated with sulphite; and with muscle proteins.

The positive nitroprusside reaction was attributed to the proteins themselves but Arnold (1910) found positive results with protein free extracts and attributed this to the presence of cysteine. However, cysteine was not isolated.

In 1921 Hopkins reported the isolation of a substance giving a positive nitroprusside test and which was undoubtedly the "Philothion" of de Rey-Pailhade (1888). (Hopkins 1921).

This substance was glutathione.

The nitroprusside reaction was conferred upon certain pure proteins when, as coagula, they were brought into contact with strong solutions of thiol compounds. This reaction was very much more intense than that produced by denaturation processes alone, (Hopkins, 1925).

It was also shown that the appearance of a sulphydryl group
on /

on treatment with glutathione was due to a reduction in the protein. Such a sulphydryl group could be oxidised by GSSG but not by molecular oxygen.

The oxidation was reversed by high concentrations of thiol, (Hopkins, 1925).

Thus the presence of disulphide and sulphydryl groups in proteins was established and subsequent investigations have been directed at the determination of the quantity of each type of group present and the functions of these groups.

The Functions of Sulphydryl and Disulphide Groups.

There has, on the whole, been little speculation about the function of protein disulphide groups. In the majority of cases it is probable that they have structural functions rather than a role in biochemical reactions.

Insulin is, as yet, the only protein in which there appears to be a direct participation of a disulphide bond in the biological function.

The reactivation of Urease by GSH, (Perlzweig, 1932) and the demonstration that sulphydryl groups were necessary for the enzymic activity of certain oxidation reduction enzymes, (Hopkins and Morgan, (1938); Rapkine, (1938) prompted investigation into the possible functions of protein sulphydryl groups.

Today there is an impressive list of enzymes, (Boyer, 1960) /

1960) which require sulphydryl groups for their activity but as yet comparatively little is known about the role of these groups in the enzymic reactions.

Even less is known about the function of sulphydryl groups in proteins which are not enzymes.

It has been suggested that the sulphydryl groups are involved in binding certain substances, such as the substrates and cofactors for enzymes; or they may act as acyl group acceptors, (Shifrin and Kaplan, 1960; Segal and Boyer, 1953).

Binding could involve an electrostatic bond between oppositely charged molecules, hydrogen bonding, or formation of a covalent bond such as a mixed-disulphide bond.

Sulphydryl groups may have a general role in maintaining proteins in a suitable configuration by the formation of intramolecular bonds, (Swenson and Boyer (1957). There is some evidence for this from studies with muscle phosphorylase, (Cecil and McPhee, 1959) and human adult haemoglobin, (Cecil and Snow, 1962).

Within the cells and tissues there is a continuous production of metabolic products which tend to oxidise sulphydryl groups. However, this does not usually happen for GSH is continuously regenerated.

It /

It is probable that the maintenance of protein sulphhydryl groups in the reduced state is the main function of GSH, (Racker, 1954; Barron, 1951).

Types of Sulphydryl Group.

Non-protein thiols react readily with nitroprusside and this property is shared by some of the proteins which contain sulphhydryl groups.

Those sulphhydryl groups which react with nitroprusside and mild oxidising agents, such as ferricyanide, are termed FREELY-REACTING sulphhydryl groups.

Other sulphhydryl groups, in the native protein, react only with more powerful reagents, such as iodine and the mercaptide-forming mercurials. These are known as SLUGGISH sulphhydryl groups.

There are, however, still other sulphhydryl groups which do not react with any of the reagents until the protein is denatured. These are the MASKED sulphhydryl groups.

It has been postulated that the sluggish and masked groups exist in the form of cyclic, reversible structures, such as thiazolines, (Linderstrom-Lang and Jacobsen, 1941).

There is no evidence to support the assumption made by Harris (1923) that the masked thiol groups are present as labile disulphide groups.

Klotz /

Klotz and Ayers, (1957) suggested that a rigid structure of water around the protein molecule masks the sulphydryl group. Denaturation disrupts the water envelope.

It is most probable that the masked sulphydryl groups are enclosed within the protein molecule and prevented from reacting with sulphydryl-reagents by the geometric configuration of the protein peptide chain, which is maintained by hydrogen bonding. Denaturation disrupts the hydrogen bonding, (Mirsky and Pauling, 1936).

These structural factors also influence the reaction of disulphide groups with reagents such as sulphite, cyanide and non-protein thiols. The accessibility of the sulphydryl groups was an important factor in the work to be described later.

The Sulphydryl-Containing Proteins Studied.

Bovine and Human Serum Albumins. Both molecules of albumin presumably exist as a single polypeptide chain as only one terminal alpha-amino-group has been found in each.

Human and Bovine serum albumins have identical sulphydryl contents in the native state. Values from a number of workers indicate the presence of approximately 0.67 sulphydryl groups per molecule, (Hughes, 1947; Hughes, 1949; Benesch and Benesch, 1948; Carter, 1959; Kolthoff et al., 1957; Klotz et al., 1958).

However, /

However, Benesch and co-workers found that titrations with silver nitrate in urea indicated one sulphydryl group per molecule of albumin, (Benesch et al., 1955).

They suggested that as serum albumin gives only a faint nitroprusside test under normal conditions yet reacts readily with iodoacetamide the increase in sulphydryl content on urea denaturation was related to the accessibility of the sulphydryl groups.

However, the variation in estimated sulphydryl content appears to depend upon the particular method used for the estimation and may be due to non-specific reaction of the reagents with the proteins, (Kolthoff et al., 1957; Benesch et al., 1956).

The evidence indicates that both human and bovine serum albumins have 0.67 sulphydryl groups per molecule or more correctly that one-third of the albumin preparation is devoid of sulphydryl groups. The fraction which contains the sulphydryl group is termed mercaptalbumin.

The properties of the non-mercapt albumin have not been studied in detail but King, in a preliminary communication, (King, 1961), states that this fraction consists of a mixture of an albumin disulphide dimer and mixed disulphides formed between the albumin and cystine, or glutathione.

The /

The albumin dimer is not formed by iodine oxidation, the sulphenic acid derivative being produced instead, (Hughes and Straessle, 1950).

However, iodine oxidation of the mercury dimer of mercaptalbumin, in which two protein molecules are linked through their sulphydryl groups by a mercury atom, does give rise to the disulphide dimer, (Straessle, 1954).

Thus it appears necessary for the albumin molecules to be orientated before the oxidation can produce a disulphide bond, and if as King (1961) reports, there is a mercaptalbumin dimer to be found in human serum it must presumably have been formed under conditions which allowed preliminary orientation of the molecules of albumin.

Human and bovine serum albumins also contain disulphide bonds. Reported values of 17 disulphide bonds per molecule for both human and bovine albumin are found, (Carter, 1959; K lthoff et al., 1958; Markus and Karush, 1957), though earlier work indicated values ranging from 14-18 disulphide bonds per molecule (see Cecil and McPhee, 1959).

The serum albumins are responsible "in vivo" for maintaining the colloid osmotic pressure and assist in the transport of bilirubin, fatty acids and drugs but it is not known if the sulphydryl group is directly involved in any of these functions.

The /

The possibility that the sulphhydryl acts as a scavenger for heavy metals has been suggested, (Cecil and McPhee, 1959).

Bovine Gamma Globulin. Bovine γ - globulin is not a homogeneous protein and consequently the sulphhydryl and disulphide content depends upon the method of preparation of the protein to some extent.

One sulphhydryl and 17 disulphide groups per molecule of bovine γ -globulin (molecular weight 150,000) have been reported, (Markus and Karush, 1957).

Sulphhydryl groups are apparently involved in the formation of serologically active macroglobulins, (Franklin et al., 1957).

The Haemoglobins. The mammalian haemoglobins have molecular weights of approximately 68,000, (Cecil and McPhee, 1959) and the globin moieties contain sulphhydryl groups, the number present varying from species to species.

All the mammalian haemoglobins so far studied have, in the molecule, four peptide chains which are not necessarily identical. These chains are not linked by disulphide bonds for these have not been found in any of the haemoglobins, (Allison and Cecil, 1958; Stein et al., 1957).

In general, dissociation of the peptide chains can be brought about by high concentrations of amides or thiols,
low /

low pH or dilution, (Gutter et al., 1956).

Thus it appears that the peptide chains are held together by three types of bond, namely, hydrogen bonds, between nitrogen, oxygen and possibly sulphur atoms; electrostatic bonds; and some form of bond involving the normally unreactive sulphydryl groups, (Cecil and Snow, 1962).

On denaturation some haemoglobins show an increase in the number of reactive sulphydryl groups, (Ingram, 1955; Allison and Cecil, 1958).

There is a great deal of variation in the values published for the sulphydryl content of the haemoglobins.

The variations within the same species seem to depend upon the reagent used and upon a misinterpretation of the results obtained.

It has been suggested, (Allison and Cecil, 1958) that the variations were due to the formation of complexes between mercaptide-forming reagents, particularly silver ions, and the protein mercaptide already formed.

When reinterpreted in the light of the suggestions made by Allison and Cecil there is good agreement between the published results, (Cecil and McPhee, 1959).

In general the best results have been obtained with organic mercurial compounds such as p-chloro-mercuribenzoate, methyl mercuric iodide and phenyl mercuric hydroxide.

The /

The values currently accepted for human adult haemoglobin are 2.2 reactive sulphydryl groups per molecule of undenatured protein and a total of 6 sulphydryl groups in denatured protein.

Bovine haemoglobin has only two sulphydryl groups whether the protein is denatured or not.

Storage of solutions of oxyhaemoglobin at 4°C results in a loss of sulphydryl content, (Ingram, 1957), whereas carboxy-haemoglobin under the same conditions does not lose any titratable sulphydryl, (Allison and Cecil, 1958).

Oxidation of the sulphydryl groups is rapid on denaturation and it has been found necessary to add an equivalent amount of mercaptide forming reagent before denaturation to avoid errors in the determination of the sulphydryl content, (Allison and Cecil, 1958).

These factors were taken into account in the work, with haemoglobins, which is to be described later.

All the haemoglobins which have been analysed for sulphydryl content have some groups which react readily in the native state. There is good evidence to suggest that these reactive groups are concerned in the reaction of haemoglobin with oxygen, (Riggs, 1953; Riggs and Wolbach, 1956; French and Edsall, 1945).

The sulphydryl groups are not concerned with the haemoglobin /

globin linkage but those groups which are unreactive in the native protein appear to be involved in labile intramolecular bonds which help to determine the extent of interaction between the haems, (Cecil and Snow, 1962).

Of the proteins used in the investigations to be described the haemoglobins are the only ones for which particular functions have been proposed for the sulphydryl groups.

Non-Protein Thiols and Disulphides. The non-protein thiol cysteine is a metabolic precursor of many proteins and glutathione. It is found, together with the sparingly soluble cystine, in low concentrations in the blood, (Krebs, 1950).

Glutathione, a tripeptide γ -glutamyl-cysteinyl-glycine, is widely distributed in animal tissues where it makes up at least 90% of the non-protein thiols.

It has been detected in only small amounts in extra-cellular fluid, (McIlwain, 1959).

Glutathione is maintained in a reduced state "in vivo" by glutathione reductase; reduced nicotin amide-adenine dinucleotide phosphate serving as the immediate hydrogen donor, (Conn and Vennesland, 1951).

Possibly the main significance of the occurrence of cysteine in glutathione, rather than in the free state, lies in the fact that glutathione is less prone to metabolic break down and /

and that the disulphide of glutathione is very much more soluble in tissue fluids than cystine.

Cysteine and GSH, and their respective disulphides were the thiols and disulphides most frequently used in the experimental work, to be described later, but on occasions thioglycollic acid, 2:3 dimercaptopropanol (BAL) and homocystine were used.

BAL and thioglycollic acid are not normal constituents of biological material but they have been much used in elucidating aspects of sulphydryl chemistry.

Homocystine is a naturally occurring disulphide which in its reduced form is involved as the methyl group acceptor in the synthesis of methionine.

The investigations which were carried out with these compounds, explored the possibilities of the interaction of non-protein thiols and disulphides with a few biologically important sulphydryl-containing proteins.

Thiol-Disulphide Interactions.

In 1925 Hopkins reported that an intense nitroprusside reaction was conferred upon coagulated pure proteins when brought into contact with strong solutions of thiols, (Hopkins, 1925). This appearance of sulphydryl groups was shown to be due to a reduction within the protein and not to adsorption of the added thiol.

The sulphydryl groups so produced, though resistant to oxidation by molecular oxygen, were oxidised by GSSG at pH 7.4.

These observations seemed to be robbed of any possible biological significance as prior coagulation of the protein was necessary to obtain the effect. However, it was shown that the nitroprusside reaction given with washed muscle protein in the native state could be abolished by GSSG and then restored by GSH.

These findings established that, by a thiol-disulphide interchange, the hydrogen atom of the sulphydryl group, whether protein or non-protein, could be transferred to a disulphide compound.

Employing cystine and thioglycollic acid in thiol-disulphide reactions with proteins, Mirsky and Anson (1935) were able to determine the sulphydryl and disulphide content of the proteins.

The /

The work of Hopkins and Morgan (1938) and Rapkine (1938) showed quite clearly that sulphydryl groups were required for the activity of certain enzymes catalysing oxidation-reduction reactions. They demonstrated what is evidently a thiol-disulphide interaction by inactivating the enzymes with GSSG. Reactivation was brought about by excess GSH.

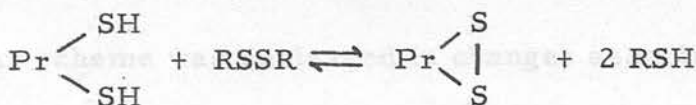
Balls and Lineweaver in studies on the enzyme papain showed that the enzyme could be inactivated by cysteine under oxidising conditions, (Balls and Lineweaver, 1939a,) or by cystine, (Balls and Lineweaver, 1939b).

This reversible inactivation of an enzyme by a non-protein disulphide is not a general property of enzymes but is found only with those enzymes which require sulphydryl groups. As far as is known only sulphydryl groups are oxidised by the added non-protein disulphide.

In a review, (Herriott, 1947) the thiol-disulphide interaction was represented as a reversible oxidation-reduction.



or



Bersin and Steudel had, however, suggested that the reaction probably proceeds through the mercaptide ion, (Bersin and Steudel, 1938).

The /

However, /

However, it was generally assumed that the mixed disulphides existed only as catalytic intermediates which could be safely neglected in biochemistry.

The reverse reaction is brought about by addition of excess thiol; various thiols differing markedly in their effectiveness.

This assumption has been shown to be incorrect for substantial amounts of mixed disulphides have been detected, and may in fact be the predominant molecular species in a reaction mixture. Previous failure to recognise this was no doubt due to the lack of suitable analytical techniques.

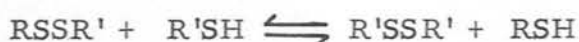
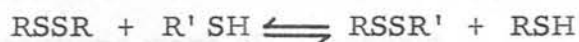
The above representation of the reaction between a thiol and a disulphide became generally accepted but it does not allow for the formation of the mixed disulphides reported in thiol-disulphide systems, (Lecher, 1920; Wikberg, 1953; Livermore and Muecke, 1954; Tabachnick et al., 1954; Lamfrom and Nielson, 1958; Stadtman, 1953).

Sulphydryl and disulphide groups have been implicated in the gel formation in protein solutions in urea and small amounts of added thiol will initiate the gel formation which had been prevented by iodoacetamide or p-chloromercuribenzoate, (Huggins et al., 1951; Frensdorff, 1953).

These authors postulated the formation of a mixed disulphide group between added thiol and a disulphide group in the protein, a sulphydryl group appearing simultaneously in concentrations of mixed disulphides were found at pH 7-9 and their rate of formation was rapid under these conditions.

A similar scheme was envisaged in changes associated with cell division in sea urchin eggs, (Mazia, 1954).

Thus the thiol-disulphide interaction came to be regarded as -



However, /

However, it was generally assumed that the mixed disulphides existed only in catalytic amounts which could be safely neglected in biochemistry.

c This assumption has been shown to be incorrect for substantial amounts of mixed disulphides have been detected, and may in fact be the predominant molecular species in a reaction mixture. Previous failure to recognise this was no doubt due to the lack of suitable analytical techniques.

Kolthoff et al., (1955) studied the interaction of cystine with thioglycollic acid and with GSH, and obtained values for the equilibrium constants of the two reactions involved in the thiol-disulphide interactions. These constants indicated the existence of large equilibrium concentrations of the mixed disulphides.

Eldjarn and Pihl (1956c) using ^{35}S -labelled compounds were able to obtain data for the systems involving GSH and cystamine or diacetylcystamine. Again high equilibrium concentrations of mixed disulphide were found at pH 7.4 and 37°C , and their rate of formation was rapid under these conditions. The mixed disulphides formed have been separated by paper electrophoresis.

A study of the reactions involved indicated that it was the mercaptide ion which reacted with the disulphide and consequently the reactions were pH dependent.

The /

The equilibrium concentration of mixed disulphide was found to be highly dependent upon the initial ratio of disulphide to sulphydryl. This finding could be of great importance in determining the extent to which mixed disulphide formation may occur under physiological conditions.

There is now evidence, (Eldjarn and Pihl (1956b) to show that similar reactions are involved in the interaction of disulphides with protein thiol groups. Using ^{35}S -labelled cystamine, Eldjarn and Pihl found that some proteins, namely, human and bovine serum albumins, human haemoglobin and catalase, bound the cystamine radioactivity. Proteins such as insulin and cytochrome C, which do not contain sulphydryl groups, did not bind the radioactivity.

The bound radioactivity could not be removed by prolonged dialysis but was removed promptly on addition of excess cysteine.

These findings suggest that cystamine is bound chemically, probably to protein sulphydryl groups. Free sulphydryl groups were shown to be necessary, for reaction of the protein with p-chloromercuribenzoate completely abolished the reaction with cystamine.

It was also shown that the total sulphydryl content of the protein fell rapidly to zero on treatment with cystamine.

"In vivo" /

"In vivo" experiments with mice have shown a substantial amount of injected radioactive cystamine to be bound to sulphhydryl containing fractions in the blood. Only minor quantities of the radioactivity are found as cystamine, cysteamine and their metabolites. The bulk of the non-protein bound radioactivity was found as a component which had the same electrophoretic mobility as synthetic cystamine-glutathione mixed disulphide, (Eldjarn and Pihl, 1956a).

There is also evidence that ingested cystamine and cysteamine become attached to circulating blood proteins in humans, (Eldjarn and Nygaard, 1954).

On the basis of the above evidence it is reasonable to assume that an equilibrium exists between cysteamine and cystamine and that the latter reacts with sulphhydryl groups "in vivo".

The ability of various sulphhydryl and disulphide compounds to react in this way will depend upon their ease of passage through cell membranes, their rate of intracellular oxidation to the disulphide form, if necessary, and their relative affinity for protein sulphhydryl groups.

It is also possible that added thiols will react with protein disulphide bonds, (Pihl and Eldjarn, 1957).

Differences in reactivity will to some extent reflect the differences /

differences in structure of the thiols and disulphides. For instance NN'-diacetyl-cystamine has been shown to react much more slowly with GSH than does cystamine, (Pihl and Eldjarn, 1957).

Eagle et al., (1960) reported the reversible binding of $\frac{1}{2}$ cystine residues by horse and human serum proteins. Protein sulphydryl groups were implicated in this binding and it was only possible to remove the cystine by heating or by treatment with inorganic, sulphur-containing reducing agents or with thiols.

The bound cystine was available as a source of sulphur to cells in tissue cultures and this seems to indicate that the cells possess a mechanism for reducing what is apparently a mixed disulphide bond.

More recently, King (1961) has reported the formation of mixed disulphides between the sulphydryl group of human serum albumin and cystine and glutathione. He suggests that the non-mercaptalbumin fraction of preparations of serum albumin consists of a mixture of two such mixed disulphides and an albumin disulphide dimer.

The formation of small amounts of a mixed disulphide between ^{35}S -labelled GSH and haemoglobin under oxidising conditions has been described by Allen and Jandl, (1961).

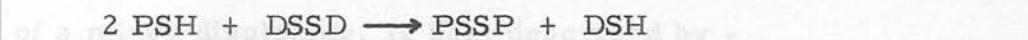
Thus /

Thus the existence of mixed disulphides involving proteins and non-protein sulphydryl and disulphide compounds has been clearly established, yet little is known of the mechanisms or stoichiometry of the reactions involved.

Klotz et al. (1958) studied the interaction of several proteins with a relatively small molecular weight disulphide compound, 2, 2', (2-hydroxy-6 sulphonaphthyl-1-azo) diphenyl disulphide (DSSD).

With bovine serum albumin, having 0.67 moles sulphydryl per mole, 0.33 - 0.37 moles DSSD were consistently bound to the protein.

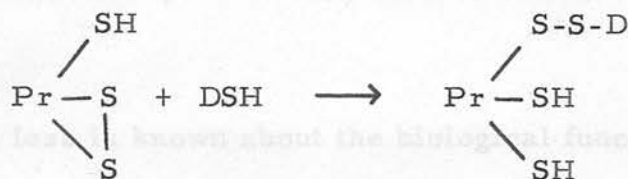
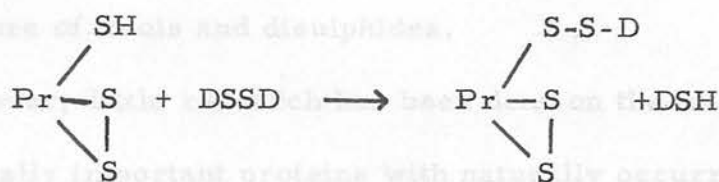
This stoichiometry seemed to indicate the reaction.



However, this implies the formation of a protein dimer with a molecular weight twice that of the original albumin.

No such dimer was detected in ultra-centrifugal studies, nor was there a change in the molecular weight determined by osmotic pressure and sedimentation measurements.

The authors finally came to the conclusion that the reaction could best be represented as -



The reaction stops when all the accessible sulphydryl groups have reacted with DSSD.

Similar results were obtained with β -lactoglobulin but with ovalbumin the reaction, while still involving the formation of a mixed disulphide, is best described by -



Ovalbumin reacts more like a simple non-protein thiol than does bovine serum albumin and β -lactoglobulin.

The wealth of evidence available establishes beyond doubt the formation of mixed disulphides by the interaction of thiols and disulphides and that protein sulphydryl and disulphide groups can participate in these interactions. Indeed it appears that the mixed disulphide may be the predominant molecular species in /

in a mixture of thiols and disulphides.

However, little research has been done on the interactions of biologically important proteins with naturally occurring thiols and disulphides in order to determine the characteristics of these interactions, which may involve mixed disulphide formation.

Even less is known about the biological function of mixed disulphides, though there has been a little speculation, (Eldjarn and Pihl, 1956c).

METHODS

GENERAL INTRODUCTION.

The high reactivity of the sulphhydryl group has led to the use of a great variety of reagents for the detection and estimation of this important chemical grouping.

In the main the methods fall into three groups depending upon the ability of the sulphhydryl group to react with (1) Oxidising agents, (2) mercaptide forming agents or, (3) alkylating agents.

The specificity of these methods depends to a large extent upon the reagent employed.

METHODS

With procedures depending upon the oxidation of the sulphhydryl group, by reagents such as iodine, ferricyanide, iodobenzoate and porphyrindia, the assumptions are made that oxidation proceeds quantitatively to disulphide and no further, that other groups are not simultaneously oxidised, and that it is sterically possible for the sulphhydryl to be oxidised to the disulphide.

Obviously there will be conditions when these assumptions will be satisfied to differing degrees, and the situation may be even more complicated in the case of a protein with a number of sulphhydryl groups of differing reactivities.

The alkylating reagents have been used extensively in studies aimed at characterising the sulphhydryl group but

METHODS

GENERAL INTRODUCTION.

The high reactivity of the sulphydryl group has led to the use of a great variety of reagents for the detection and estimation of this important chemical grouping.

In the main the methods fall into three groups depending upon the ability of the sulphydryl group to react with (1) Oxidising agents, (2) mercaptide forming agents or, (3) alkylating agents.

The specificity of these methods depends to a large extent upon the reagent employed.

With procedures depending upon the oxidation of the sulphydryl group, by reagents such as iodine, ferricyanide, iodosobenzoate and porphyrindin, the assumptions are made that oxidation proceeds quantitatively to disulphide and no further, that other groups are not simultaneously oxidised, and that it is sterically possible for the sulphydryl to be oxidised to the disulphide.

Obviously there will be conditions when these assumptions will be satisfied to differing degrees, and the situation may be even more complicated in the case of a protein with a number of sulphydryl groups of differing reactivities.

The alkylating reagents have been used extensively in studies aimed at characterising the sulphydryl group but relatively /

relatively little in the determination of sulphhydryl content.

The reagents which have been used for alkylation are of two types, firstly those containing an "active" halogen atom, such as iodoacetate, and secondly those compounds which possess an "active" double bond, such as N-ethyl maleimide.

Electrometric methods for sulphhydryl estimation have been developed and the procedure for amperometric titration first applied by Kolthoff and Harris, (1945) was subsequently used by Benesch and Benesch, (1948) for the determination of sulphhydryl groups in biological materials, including proteins.

These methods depend upon the formation of an undissociated mercaptide between a sulphhydryl group and a heavy metal ion.

Combination of the heavy metal ions with groups other than sulphhydryl, and with the mercaptides already formed constitute the chief sources of error but these errors can largely be avoided by suitable control of the conditions under which the estimations are carried out.

The heavy metal ions most commonly employed are those of silver and mercury, the latter having a greater specificity than silver as there is less tendency for excess mercuric ions to form complexes with the mercaptide already formed.

However, the divalency of the mercuric ion can give rise to /

to ambiguities and this has led to the use of compounds of the general type, RHgX ; where R represents a simple alkyl or aryl grouping and X is a halogen atom, or some other substituent such as hydroxyl or nitrate.

The ability of sulphydryl groups to form mercaptides has been made use of by Boyer, (1954), who measured spectrophotometrically the increase in absorption in the region of 255 $\text{m}\mu$ which occurred when a mercaptide was formed between p-chloromercuribenzoate (pCMB) and a sulphydryl compound.

The magnitude of this increase was proportional to the sulphydryl content of the compound.

Estimation of Disulphides. The estimation of compounds with disulphide bonds involves the reduction of these bonds to give sulphydryl groups which are measured by one of the above methods.

Reduction has been brought about by reagents such as sulphite, cyanide, borohydride and zinc with hydrochloric acid; or by electrolytic reduction as described by Dohan and Woodward, (1939).

In the case of oxidised glutathione, reduction has been brought about by the specific enzyme glutathione reductase, (Thomson and Martin, 1959).

Estimation of Protein Sulphdryl and Disulphide Content.

Analysis /

Analysis of proteins for their sulphydryl and disulphide content presents some special problems.

In much of the earlier work published on the sulphydryl and disulphide content of proteins the values obtained were based on the analysis of the products of acid hydrolysis of the protein. The cysteine and cystine released by hydrolysis have been estimated in various ways but it has been shown that both amino acids are decomposed to some extent during the acid treatment.

Therefore, with analyses of protein, it is desirable to use intact protein, either native or denatured, to obtain values for sulphydryl and disulphide content.

Reference has been made earlier to the different reactivities exhibited by the various types of sulphydryl group and these differences in activity are important when analyses of sulphydryl content are being carried out.

Conditions must be carefully controlled to prevent ambiguities.

In general the organic mercurials of the type RHgX have been found to be the most useful for protein sulphydryl estimations, (Cecil and McPhee, 1959).

PREPARATION OF PROTEINS.

The BSA (Fraction V) and bovine γ globulin (Fraction II) were obtained from Armour Biochemicals Ltd., Eastbourne.

The HSA (Fraction V) was kindly presented by the Blood Transfusion Service, Royal Infirmary, Edinburgh.

These protein fractions were prepared by the Cohn procedure, (Cohn et al., 1946).

In the first instance a dried preparation of human haemoglobin was obtained by dialysis of an haemolysate of pooled, washed erythrocytes against 2.8 M phosphate buffer, pH 6.8 and drying the precipitate formed over calcium chloride and phosphorus pentoxide, (Drabkin, 1949).

Stored at 4°C, in the dry state, this haemoglobin preparation lost a considerable amount of the titratable sulphdryl groups.

Haemoglobin preparations, suitable for study of the sulphdryl groups were therefore prepared by the procedure described by Allison and Cecil, (1958).

Erythrocytes from human or bovine sources were washed three times with 0.9% NaCl after separation from the plasma.

The cells were then haemolysed by the addition of an equal volume of glass distilled water, followed by repeated freezing and thawing.

The haemolysate was dialysed against deionised water at /

at 4°C for two days, with three changes of the deionised water.

As much as possible of the stroma, and any other insoluble matter, was removed by centrifugation at 1000 g. for half an hour.

The haemoglobin concentration and the sulphdryl content of the preparation were measured before storing at - 16°C in aliquots suitable for daily use.

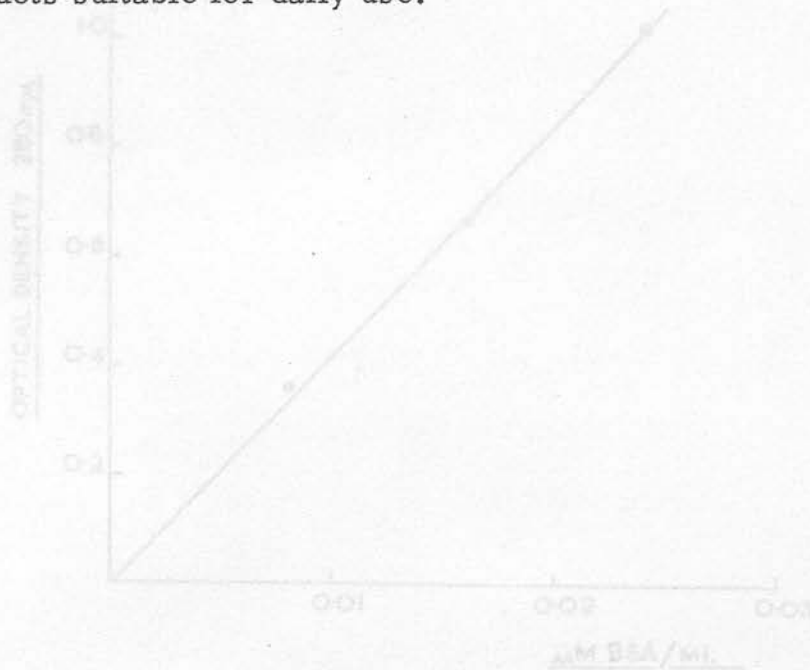


Fig. M. 1

Relationship of Optical Density at 280 m μ to BSA
Concentration

0.05, 0.10, 0.15 ml. BSA solution (0.8 μ M BSA per ml.) diluted to 5 ml. with distilled water.
Optical densities measured at 280 m μ .

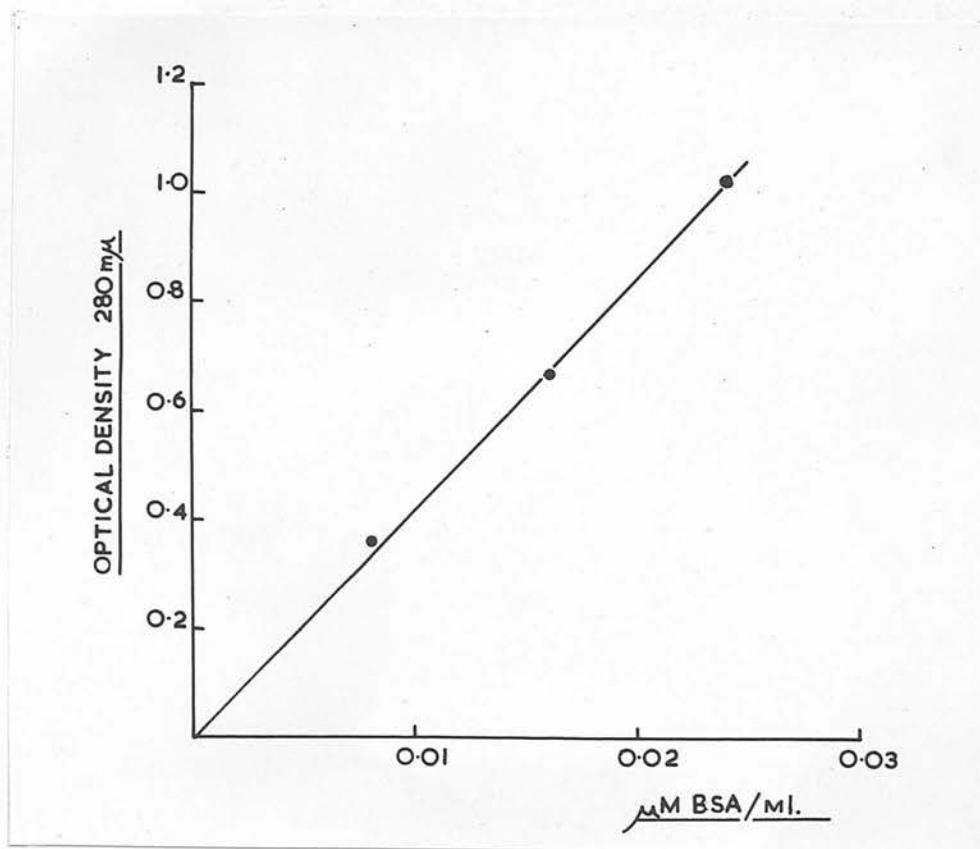
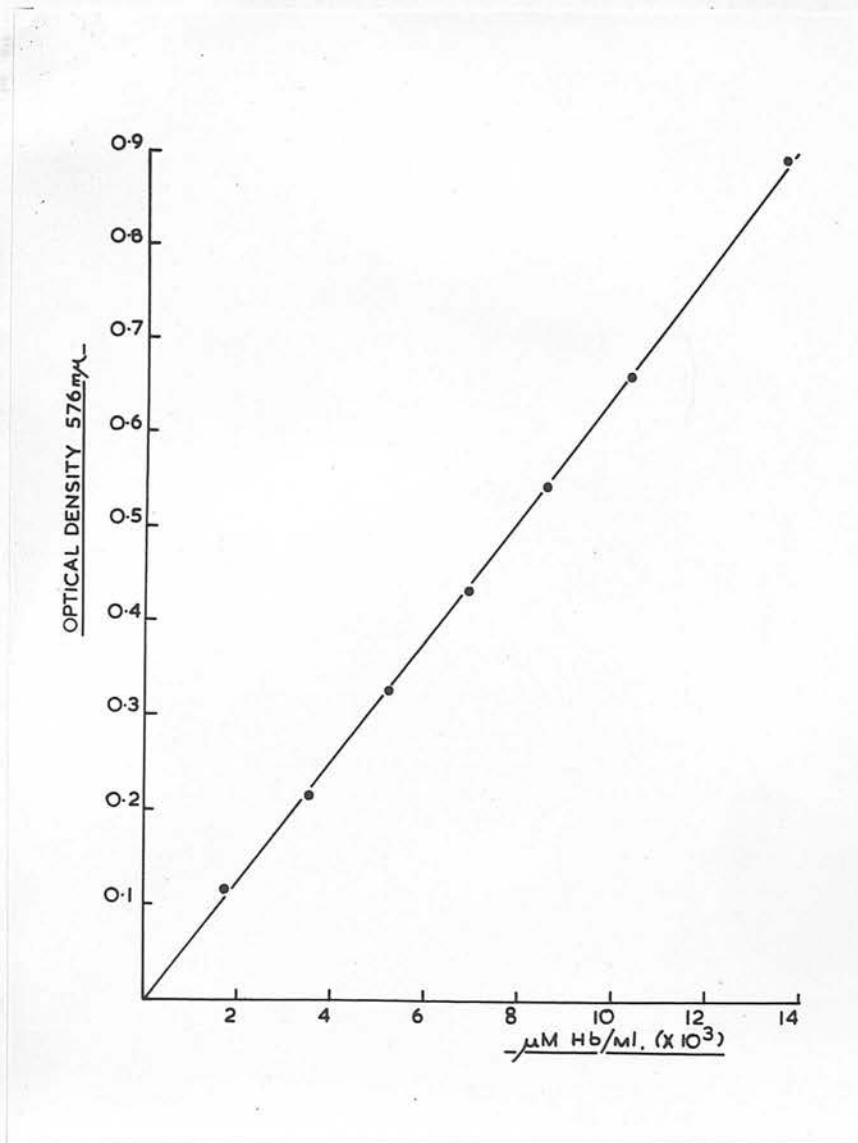


Fig. M.2

Oxyhaemoglobin Standards

(See Table M. 1)



Estimation of Protein Concentrations.

TABLE M.1.

Albumin. The concentration of albumin in solution was

OXYHAEMOGLOBIN STANDARDS IN 0.1% AMMONIA

Blood of known Hb content diluted with 0.1% ammonia and the optical densities of the solutions measured at 576 mμ

Serial dilutions of an albumin solution made up in distilled

water from a accurately weighed, dry specimen of crystalline

| Haemoglobin Concentration (μm Hb/ml) | Optical Density at 576 mμ |
|--|------------------------------|
|--|------------------------------|

Haemoglobin. Haemoglobin was estimated as oxyhaemoglobin

| | |
|--------|-------|
| 0.0017 | 0.115 |
|--------|-------|

at 576 mμ, in dilute ammonia.

| | |
|--------|-------|
| 0.0035 | 0.214 |
|--------|-------|

A volume of haemoglobin solution containing approximately

| | |
|--------|-------|
| 0.0052 | 0.326 |
|--------|-------|

0.01 μm Hb per ml. was diluted to 10 ml. with 0.1% ammonia

| | |
|--------|-------|
| 0.0069 | 0.433 |
|--------|-------|

and the optical density of the solution measured at 576 mμ.

| | |
|--------|-------|
| 0.0086 | 0.540 |
|--------|-------|

The standard curve (Fig. M.2) (Table M.1.) was obtained

| | |
|--------|-------|
| 0.0104 | 0.658 |
|--------|-------|

using dilutions of a blood of known haemoglobin content obtained

| | |
|--------|-------|
| 0.0138 | 0.890 |
|--------|-------|

from C. Davis Keeler Ltd. (London).

Purification of PCMB. The p-chloromercuribenzoic acid

obtained from commercial sources was purified by dissolving

in alkali and reprecipitating by the addition of acid (Hallerman,

1943).

The p-chloromercuribenzoic acid was dissolved in the

minimum amount of 1N NaOH and any residue removed by

centrifugation.

Complete /

Estimation of Protein Concentrations.

Albumin. The concentration of albumin in solution was determined from measurements of the optical density at 280m μ of suitably diluted solutions, (Cohn et al., 1947).

Serial dilutions of an albumin solution made up in distilled water from a carefully weighed, dry specimen of crystalline bovine serum albumin and the standard curve, (Fig. M.1.) obtained.

Haemoglobin. Haemoglobin was estimated as oxyhaemoglobin at 576 m μ , in dilute ammonia.

A volume of haemoglobin solution containing approximately 0.01 μ m Hb per ml. was diluted to 10 ml. with 0.1 % ammonia and the optical density of the solution measured at 576 m μ .

The standard curve (Fig. M.2) (Table M.1.) was obtained using dilutions of a blood of known haemoglobin content obtained from C. Davis Keeler Ltd. (London).

Purification of PCMB. The p-chloromercuribenzoic acid obtained from commercial sources was purified by dissolving in alkali and reprecipitating by the addition of acid (Hellerman, 1943).

The p-chloromercuribenzoic acid was dissolved in the minimum amount of 1N NaOH and any residue removed by centrifugation.

Complete /

Complete precipitation of the p-chloromercuribenzoic acid was brought about by addition of INHCl .

The precipitate obtained was washed thoroughly with distilled water until free of HCl and the whole procedure repeated twice.

The final precipitate was dried at 110°C and the dried product crushed to a fine powder and stored over calcium chloride at 4°C .

pCMB solutions were obtained by dissolving a weighed amount of the acid in the minimum amount of 0.1 N. NaOH and diluting to the desired volume with water.

The purity of the acid was determined by the addition of an excess of standard iodine to a pCMB solution, followed by titration of the unreacted iodine with standard sodium thiosulphate solution, (Boyer, 1954).

If the acid was not 100% pure by this assay the precipitation procedure was repeated.

Routine standardisation of the pCMB solutions was done spectrophotometrically at 255 $\text{m}\mu$.

No significant change in absorption took place over a period of weeks when the solutions were stored at 4°C .

A METHOD FOR THE ESTIMATION OF PROTEIN SULPHYDRYL CONTENT.

Boyer, (1954) described a method for the spectrophotometric determination of the sulphydryl content of protein and non-protein substances.

The described method is both rapid and sensitive; properties which make it particularly useful in reaction rate studies.

The procedure is based upon the increase in optical density in the region of 250 m μ accompanying the formation of a mercaptide between sodium p-chloromercuribenzoate, or other similar mercurials, and the sulphydryl group of the compound being studied.

Boyer found that this increase in absorption is proportional to the sulphydryl content of the compound.

Measurements are made in the region of 250 m μ as at, or near, this wavelength the increase in absorption on mercaptide formation is large compared with the increase at the wavelength of maximum absorption in the region of 230 m μ . The actual wavelength at which the maximum effect is found moves towards the visible with a decrease in pH.

pCMB can react slowly with groups in the protein other than sulphydryl groups but it is only reaction with the sulphydryl groups which results in the large increase in absorption near /

near 250 m μ .

This simple spectrophotometric method seemed to be ideal for the study of the reactions of proteins with thiols and disulphides.

Unfortunately there was considerable absorption at, and around, 250 m μ due to the proteins which were being studied and this absorption was great in comparison to the increase in absorption found on mercaptide formation.

For instance, the absorption at 255 m μ due to an albumin solution (0.16 μ M per ml) was 0.980, while the increase in absorption on mercaptide formation, following the addition of pCMB, was only 0.180.

Development of the Method.

The background absorption due to the protein limits the sensitivity of the method and a new technique has now been developed which overcomes this obstacle.

The principle of the new method is to precipitate the protein after reaction with a known amount of pCMB and to estimate the remaining pCMB spectrophotometrically.

This differs from Boyer's method in the following ways -

1. There is no absorption due to protein.
2. Instead of measuring the increase in optical density on mercaptide formation the pCMB-protein mercaptide is precipitated and the unreacted pCMB estimated.

Fig. M. 3

Effect of pH on Absorption Spectrum
of pCMB

A. pCMB solution, pH 7.4

B. pCMB in metaphosphoric acid.

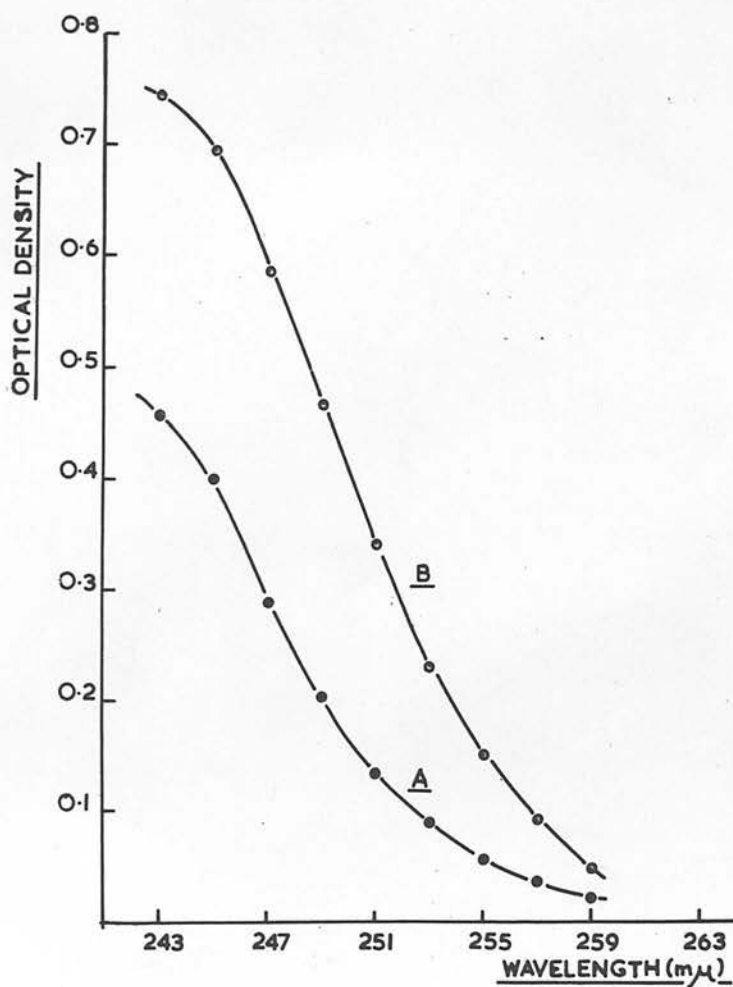
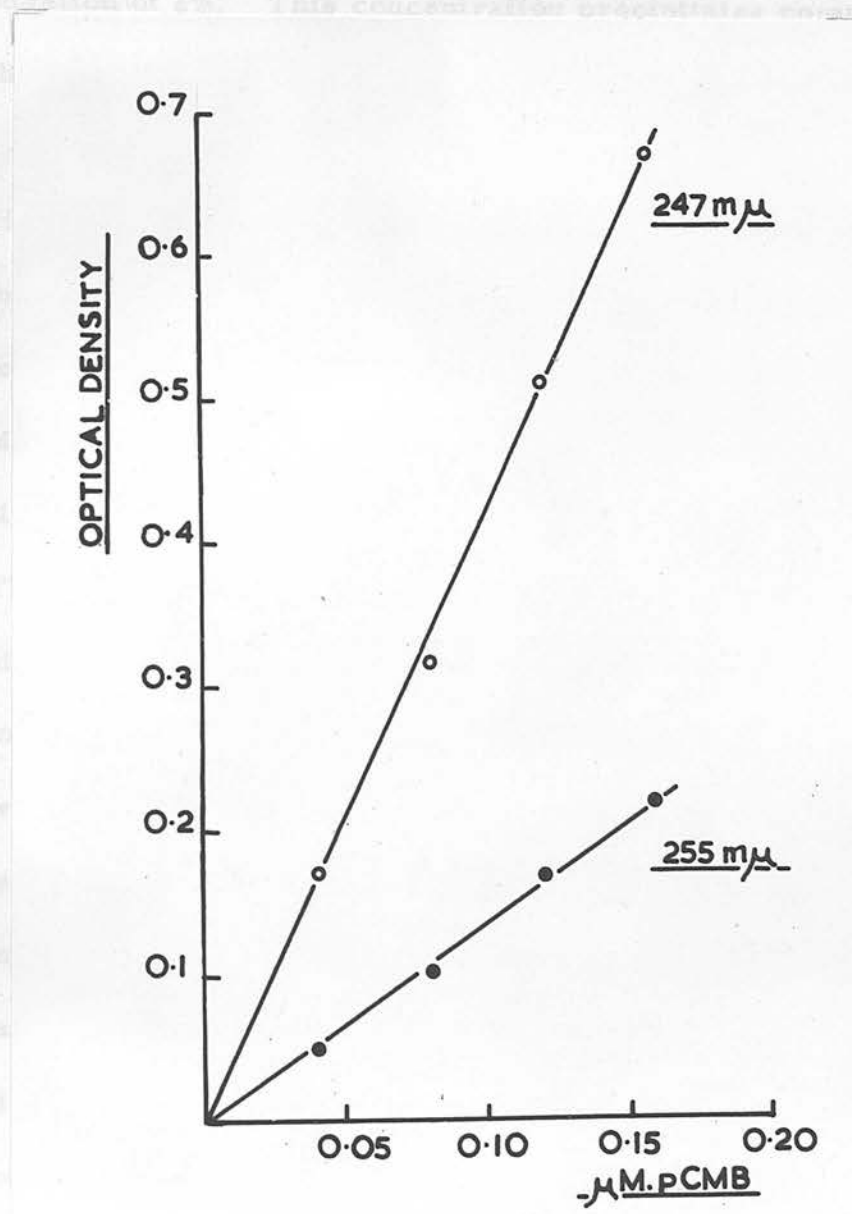


Fig. M.4

Graph showing linear relationship of absorption to concentration of pCMB in 2% metaphosphoric acid.



3. The estimation is carried out in acid solution.

It was found that the most convenient way of precipitating the protein was by the addition of metaphosphoric acid to a final concentration of 2%. This concentration precipitates completely even the largest amounts of protein used, leaving a clear supernatant on centrifugation.

However, pCMB is very insoluble in the acid conditions and tends to precipitate from solution, but prior addition of acetate or phosphate buffer, pH 7.4, to a final concentration of 0.03 M, overcomes this difficulty without affecting the protein precipitation.

The displacement of the absorption spectrum of pCMB, brought about by the addition of metaphosphoric acid, has the effect of doubling the absorption of a given amount of pCMB at the wavelength used (Fig. M.3), thus increasing the sensitivity of the estimations.

In the experiments which follow the arbitrarily chosen wavelength of 247 m μ was used. At this wavelength the absorption due to the pCMB is approximately three times that obtained for the same solution at 255 m μ (Fig. M.3).

Fig. M.4 shows that the absorption due ^{to} pCMB increases linearly with increase in the concentration of the mercurial.

When /

When a sulphydryl-containing protein was treated with pCMB, in a buffered medium and the proteins subsequently precipitated, the determination of the optical density showed that the quantity of pCMB removed from solution was proportional to the concentration of the protein initially present, (Table M.2 and Fig. M.5).

However, the pCMB could have been removed, in part, by reaction with groups other than sulphydryl groups or non-specifically by occlusion during the protein precipitation.

As the pCMB which has a low affinity for groups other than sulphydryl, was never present in large excess and was only in contact with the protein for short periods of time, non-specific reaction was considered unlikely.

The possibility of removal of the pCMB by physical forces during precipitation was tested by treating the albumin with iodine, in the way described by Hughes and Straessle, (1950), in order to oxidise the sulphydryl groups completely but to involve other groups in the protein (e.g. tyrosyl, histidyl and tryptophanyl) as little as possible.

This was done by reaction of 10.08 moles iodine with 1 mole of albumin at pH 10.06 for 2 days at 4°C.

After removal of the excess iodine by dialysis, aliquots of the iodinated albumin were then treated with pCMB and the protein /

Fig. M.5

Effect of Iodination of Albumin upon
the removal of pCMB

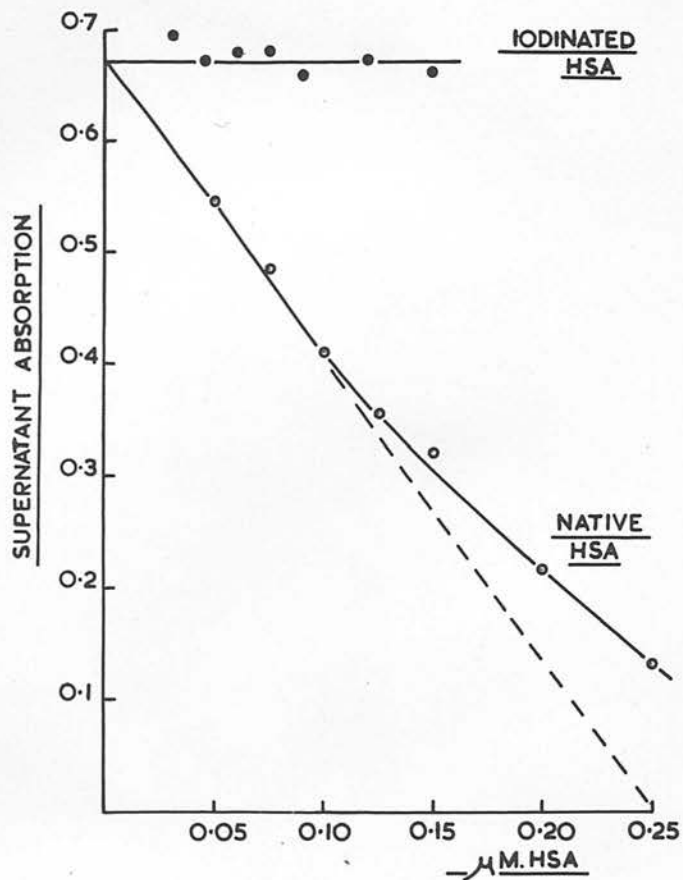


TABLE M.2.

EFFECT OF IODINE OXIDATION ON ABILITY OF
ALBUMIN TO REMOVE pCMB FROM SOLUTION

Aliquots of H.S.A. solution (1 μ m/ml) reacted with 0.16 μ m pCMB at pH 7.4 in a total volume of 2 mls. Made to 2% metaphosphoric acid to precipitate protein. Final volume 3 mls. Supernatants read at 247 m μ .

OPTICAL DENSITY AT 247 m μ
OF SUPERNATANTS

| <u>Protein Concentration (μm/3 mls.)</u> | <u>Untreated HSA</u> | <u>Iodinated-HSA</u> | <u>pCMB Standard</u> |
|---|----------------------|----------------------|--------------------------|
| 0 | - | - | 0.680 |
| 0.030 | - | 0.696 | |
| 0.045 | - | 0.669 | |
| 0.050 | 0.546 | - | |
| 0.060 | - | 0.680 | |
| 0.075 | 0.488 | 0.682 | |
| 0.090 | - | 0.659 | |
| 0.100 | 0.410 | - | |
| 0.120 | - | 0.673 | |
| 0.125 | 0.355 | - | |
| 0.150 | 0.322 | 0.664 | |
| 0.200 | 0.215 | - | |
| 0.250 | 0.131 | - | |

protein subsequently precipitated.

The results shown in Table M.2 and Fig. M.5 were obtained.

These findings indicate that no pCMB was removed from solution on precipitation of the protein and it was concluded that sulphhydryl groups are necessary for the removal of the pCMB.

Therefore it is possible to calculate the sulphhydryl content of a protein solution from spectrophotometric measurements of the amount of pCMB remaining in the acid supernatant, readings being made against the appropriate 'blank' solution to eliminate background effects due to the reagents.

It can be seen from Fig. M.5 that with human serum albumin (HSA) there is a decrease in the relative degree of removal of pCMB as the albumin concentration becomes greater. This effect is also seen with bovine serum albumin (BSA) but not with the haemoglobin preparations.

No complete explanation for this can be given but it appears to be a result of an equilibrium set up within the reaction mixture.

From the degree of removal of pCMB over the linear portion of the curve the sulphhydryl content of the HSA was calculated to be $0.65 \mu\text{M}$ sulphhydryl per μM albumin, taking the molecular weight of the protein as 65,000, (Hughes, 1954).
BSA /

BSA had an identical sulphhydryl content.

These values are in close agreement with those reported by Carter, (1959), Hughes, (1947) and several other workers. Possible reasons for the fractional sulphhydryl content are discussed in the introduction to this thesis (p. 7).

Hughes (1949) has isolated mercaptalbumin, the sulphhydryl-containing fraction, using mercuric chloride. Attempts were now made to separate the non-sulphhydryl albumin from the mercaptalbumin by a new method.

An anion exchange resin, Amberlite IRA 400 (OH) was converted first to the chloride form and then reacted with sodium p-chloromercuribenzoate.

The resin in this form was able to bind the non-protein thiol glutathione. However, albumin was not bound to the resin but in fact removed the mercuribenzoate, which was detectable in the eluants by the dithizone test for mercury compounds.

No further attempts were made to remove the non-~~mercap~~-talbumin fraction.

The Reproducibility of the Method.

This is illustrated by the values for the sulphhydryl content of BSA obtained in ten consecutive determinations on the same protein solution (Table M. 3).

From /

TABLE M. 3.

REPRODUCIBILITY OF ESTIMATIONS
OF SULPHYDRYL CONTENT OF BSA

0.14 μ M BSA reacted with 0.16 μ M pCMB in 0.05 M phosphate buffer pH 7.4. Total volume 2 mls. Metaphosphoric acid added to final concentration of 2% to precipitate protein: Supernatants read at 247 m μ .

| | <u>Optical density</u> <u>247 mμ</u> | <u>Sulphydryl content</u> (moles SH per mole BSA) |
|--------------------------------|--|---|
| pCMB standard | 0.650 | - |
| Protein solutions | | |
| 1 | 0.278 | 0.652 |
| 2 | 0.281 | 0.649 |
| 3 | 0.268 | 0.670 |
| 4 | 0.271 | 0.664 |
| 5 | 0.269 | 0.670 |
| 6 | 0.289 | 0.632 |
| 7 | 0.291 | 0.630 |
| 8 | 0.271 | 0.664 |
| 9 | 0.276 | 0.656 |
| 10 | 0.289 | 0.632 |
| Mean \pm Standard Deviation) | 0.278 \pm 0.009 | 0.652 \pm 0.016 |
| Range | 0.268 - 0.289 | 0.630 - 0.670 |

From determinations carried out on different solutions at various times the mean value for the sulphhydryl content was found to be 0.657 ± 0.023 (\pm S.D.) μM sulphhydryl per μM BSA.

It was found that BSA which had been recrystallised (Crystalline Bovine Plasma Albumin. Armour and Company) gave a value of $0.82 \mu\text{M}$ sulphhydryl per μM BSA. A similar phenomenon has been described by Boyer (1954).

Details of the method finally adopted.

Reagents.

6% Metaphosphoric acid in distilled water.

8×10^{-4} M sodium p-chloromercuribenzoate.

Prepared by dissolving 28 mgm p-chloromercuribenzoic acid in 0.01N sodium hydroxide, and making volume to 100 ml.

0.2M phosphate buffer, pH 7.4, prepared by mixing 0.2M Na_2HPO_4 (81ml) with 0.2M $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ (19 ml).

Procedure.

To a protein solution containing approximately $0.1 \mu\text{M}$ sulphhydryl was added 0.2M phosphate buffer, pH 7.4 (0.5ml) and 8×10^{-4} M sodium p-chloromercuribenzoate (0.2ml. $0.16 \mu\text{M}$). The volume was made up to 2 ml. with distilled water and stood for 10 minutes after mixing. The protein was then precipitated by addition of 6% metaphosphoric acid (1 ml.) and the precipitate /

precipitate removed by centrifugation.

The optical density of the clear supernatant was measured in a Unicam SP.500 spectrophotometer at 247 mμ in silica cuvettes of 1 cm. light path.

The spectrophotometer readings were made against a supernatant 'blank' containing distilled water (0.2 ml.) instead of the pCMB.

The optical density of a pCMB standard solution, containing 0.16 μM pCMB in 3 ml. was also measured.

From these measurements the sulphydryl content of the protein could be determined, either graphically by extrapolation of the linear portion of the curve, obtained by reacting increasing volumes of a protein solution with a fixed amount of pCMB, to cut the abscissa at a point equivalent to the amount of sulphydryl protein required to remove all the pCMB (Fig. M.5); or by insertion of the appropriate values in the equation:-

$$\frac{(\text{O.D. pCMB standard} - \text{O.D. Protein Supernatant})}{\text{O.D. pCMB standard}} \times \frac{(\text{pCMB})}{(\text{protein})} = \text{sulphydryl content of protein. (in } \mu\text{M SH per } \mu\text{M protein)}$$

O.D. represents the optical density of the solutions at 247mμ;

(pCMB) the concentration of pCMB, in μM, initially present;

and (protein) the concentration of protein, in μM, initially present.

The Sulphydryl Content of Other Proteins.

In addition to the human and bovine serum albumins the

pCMB /

TABLE M.4

EFFECT OF IODINE OXIDATION OF BOVINE- γ -
GLOBULIN ON THE REACTION WITH pCMB

Aliquots of bovine- γ - globulin solution reacted with 0.16 μ M pCMB in 0.05 M phosphate buffer, pH 7.4. Total volume 2 mls. Protein precipitated by addition of 1 ml. metaphosphoric acid. Supernatants read at 247 m μ .

OPTICAL DENSITY AT 247 m μ
OF SUPERNATANTS

| <u>Protein</u> <u>Concentration</u> (μ M/3mls) | <u>Untreated</u> <u>Bovine-γ-globulin</u> | <u>Iodinated</u> <u>Bovine-γ-globulin</u> | <u>pCMB</u> <u>standard</u> |
|---|--|--|--------------------------------|
| 0 | - | - | 0.656 |
| 0.04 | 0.649 | 0.660 | |
| 0.08 | 0.598 | 0.682 | |
| 0.12 | 0.545 | 0.620 | |
| 0.16 | 0.500 | 0.659 | |
| 0.20 | 0.460 | 0.670 | |

pCMB method for sulphydryl estimation was applied to several other proteins.

Bovine- γ -Globulin.

This protein reacted with pCMB to an extent indicating a sulphydryl content of 0.27 moles sulphydryl per mole of protein, taking the molecular weight of the protein to be 156,000, (Markus and Karush, 1957).

The reaction of the protein with pCMB was abolished by treatment with iodine at 4°C and pH10 (Table M. 4)

Haemoglobins.

Two haemoglobin preparations, from different species, were analysed for sulphydryl content.

(1) Human haemoglobin (HHb). The currently accepted values for HHb indicate a content of 2.2 moles sulphydryl per mole HHb for the undenatured protein and 6 moles sulphydryl per mole HHb for the denatured protein, (Cecil and McPhee, 1959).

HHb has a molecular weight of 68,000, (Cecil and McPhee, 1959).

The pCMB method gave values in the region of 6 sulphydryl groups per molecule, pCMB reacting with the sulphydryl groups released on denaturation of the HHb with metaphosphoric acid.

The /

The mean value obtained from five single determinations was 6.1 moles sulphhydryl per mole HHb. (Standard deviation ± 0.2).

(2) Bovine haemoglobin. Bovine haemoglobin does not release any sulphhydryl groups on denaturation, (Cecil and McPhee, 1959).

The mean value obtained from thirteen single determinations of the sulphhydryl content of bovine haemoglobin by the pCMB method was 2.0 ± 0.1 (\pm S.D.) moles sulphhydryl per mole haemoglobin.

This value is in good agreement with that reported by Murayama, (1958).

Treatment of the haemoglobin with iodine did not appear to remove all the sulphhydryl groups, approximately half of the original remaining after the oxidation.

Increasing the amount of iodine had no further effect. In both cases there was some precipitation of insoluble matter which made interpretation of the effect impossible.

Sensitivity of the pCMB method.

The pCMB method as developed is most suited to the estimation of the sulphhydryl content of BSA or HSA.

The haemoglobins with their greater sulphhydryl content can /

TABLE M.5

| | <u>Protein sulphydryl</u> <u>content</u> (μ MSH per μ M protein) | <u>Standard</u> <u>Deviation</u> | <u>Range</u> | <u>No. of</u> <u>Determinations</u> |
|-------------------------|---|-------------------------------------|--------------|--|
| Bovine serum albumin | 0.66 | \pm 0.023 | 0.62 - 0.69 | 20 |
| Human haemoglobin | 6.1 | \pm 0.2 | 5.8 - 6.3 | 5 |
| Bovine haemoglobin | 2.0 | \pm 0.1 | 1.8 - 2.2 | 13 |

can not be analysed by this method with the same accuracy as the albumins.

In the haemoglobin sulphydryl analysis by the pCMB method small differences in spectrophotometer readings, which in themselves are insignificant, give rise to considerable differences in the apparent sulphydryl content.

This is especially true of human haemoglobin which has a total of six sulphydryl groups per molecule. These differences in sensitivity are reflected in the range of values shown in

Table M. 5.

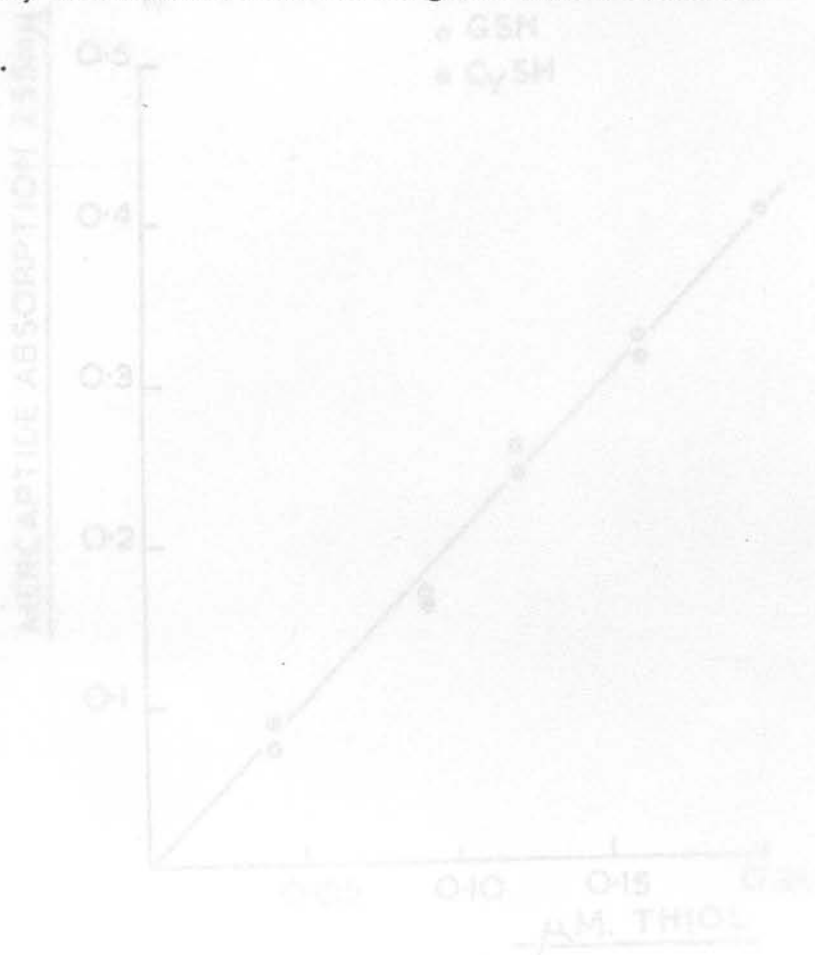


Fig. M.6

Increase in Optical Density Due to Mercaptide Formation

0.20 μM pCMB reacted with thiol (0-0.20 μM) in total volume 3 ml, containing 2% metaphosphoric acid and 0.2mM phosphate buffer pH 7.4. Optical density measured at 255 m μ . Absorption due to mercaptide formation found by subtracting from these measurements the optical density of a solution containing 0.20 μM pCMB and No Thiol.

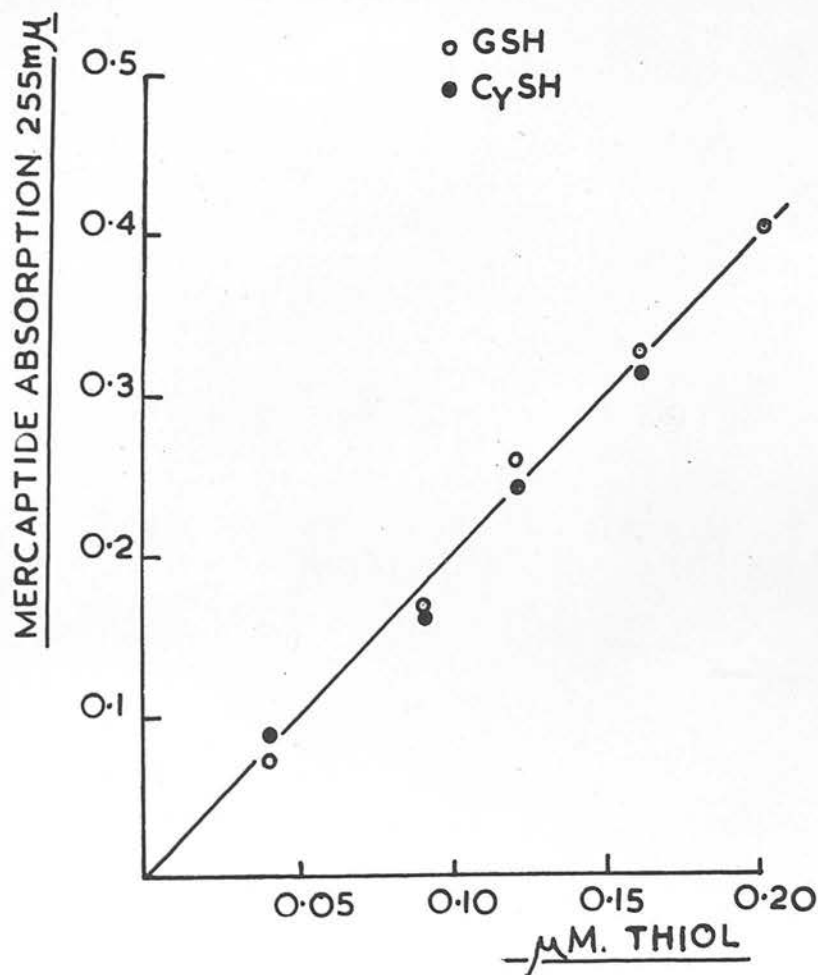


TABLE M. 6

GSH AND CySH OPTICAL DENSITY INCREASE
ON MERCAPTIDE FORMATION

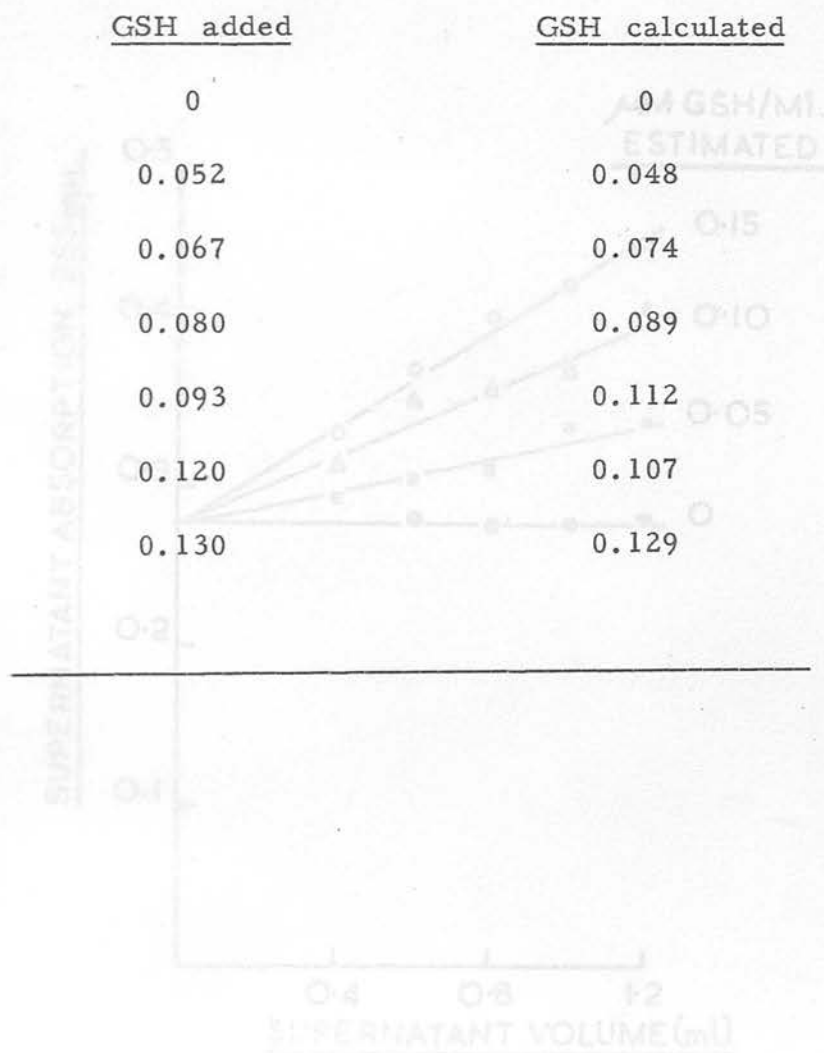
(Details of procedure in Fig. M. 4)

| <u>μM Thiol</u> <u>Added to 0.2μM pCMB</u> | <u>Optical Density increase at 255 mμ.</u> | |
|---|--|------------------|
| | <u>with GSH</u> | <u>with CySH</u> |
| 0.04 | 0.069 | 0.089 |
| 0.08 | 0.170 | 0.159 |
| 0.12 | 0.261 | 0.242 |
| 0.16 | 0.331 | 0.317 |
| 0.20 | 0.409 | - |

TABLE M. 7

GSH ESTIMATION IN THE PRESENCE OF GSSG

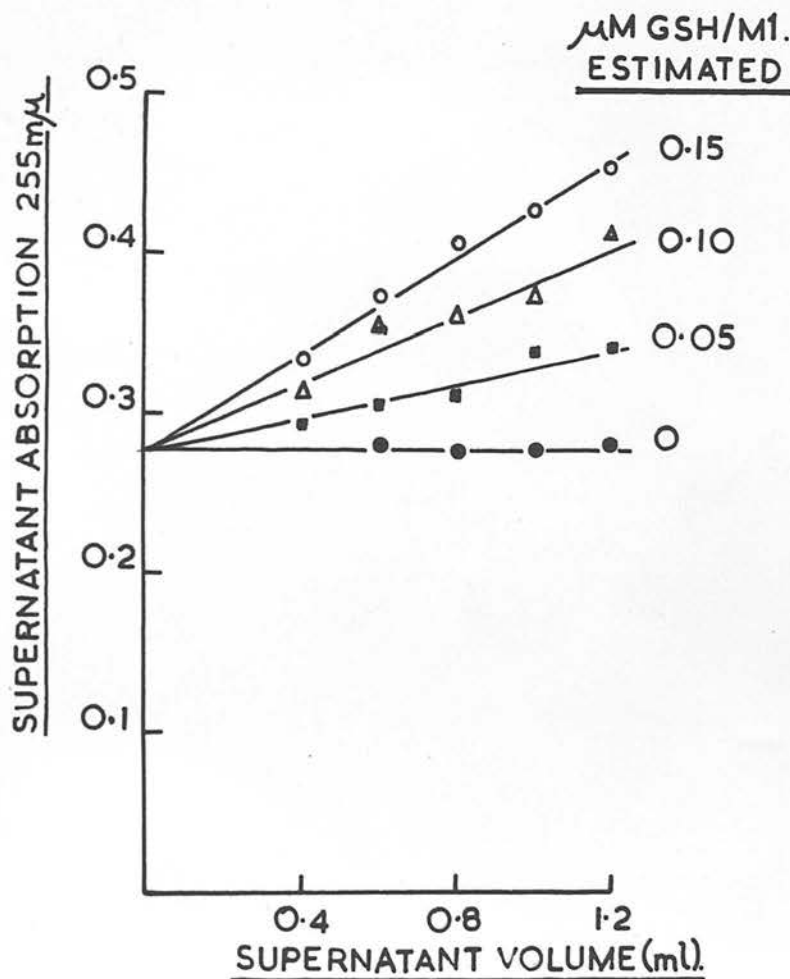
Mixtures of 0 - 0.8 μ M GSH with 0.8 μ M GSSG in total volume 6 mls. containing 0.8 μ M pCMB
Optical density measured at 255 m μ and GSH calculated from increase in absorption. (Fig. M.4)



pCMB Method:- The non-protein disulfide, glutathione (GSH) and

Recovery of GSH from mixtures of GSH and BSA

0-0.9 μ M GSH with 1 μ M BSA in total volume 6 ml. BSA immediately precipitated. Estimation of GSH in aliquots of supernatants by the pCMB method. Mixtures initially contained:- 0, 0.05, 0.10 and 0.15 μ M GSH per ml. respectively.



The Estimation of Non-Protein Sulphydryl Compounds.

pCMB Method:- The non-protein thiols, glutathione and cysteine, were estimated by the method described by Boyer, (1954).

The increase in absorption due to the formation of the thiol-mercuribenzoate mercaptide was measured at 255 m μ in acid solution, after the precipitation of proteins, or in neutral, protein-free solutions.

The increase in absorption on mercaptide formation was shown to be linearly related to the amount of thiol present, (Fig. M.6 and Table M.6) and the presence of GSSG does not interfere with the determination of the thiol (Table M.7).

The recovery of GSH added to protein, which was immediately precipitated, was of the order of 100%; calculating the GSH content from Fig. M.6 for any given increase in absorption (Fig. M.7).

Reagents:- These were the same as those used for the estimation of protein sulphydryl content (see page 41).

Standard solutions of the thiols (1 μ M per ml.) were made up freshly before use.

Procedure:- To 2 ml. solution, either containing protein or protein free, but containing up to 0.32 μ M thiol, was added 6% metaphosphoric acid (2 ml.).

After /

After removing any protein precipitate by centrifugation a 2 ml. aliquot of the supernatant was mixed with 8×10^{-4} M pCMB (0.2 ml. $0.16 \mu\text{M}$) in 0.2 M phosphate buffer pH 7.4, (0.5 ml.). The final volume was made to 3 ml. with distilled water.

The optical density of the solutions containing the mercaptide was determined at 255 m μ . against a reagent blank containing no pCMB. A pCMB standard containing no thiol was also read at 255 m μ .

Several dilutions of the thiol containing solutions were usually made and a series of measurements of optical density obtained. (E.g. Fig. M.7.).

Failure to buffer the pCMB solution before the addition of the acid supernatant resulted in the precipitation of the pCMB (see p. 35).

In protein free solutions the addition of metaphosphoric acid could be omitted.

The method is of course not specific and pCMB will react with any thiol but in these studies only one sulphydryl compound was present at any one time during the estimations.

Particular use was made of this method in testing the efficacy of dialysis in removing non-protein thiols from protein solutions.

The Alloxan '305' Method:- /



The Alloxan '305' Method:- GSH was also estimated by a modification of the Alloxan '305' method described by Patterson and Lazarow, (1955).

In this method GSH reacts with excess alloxan giving a product which has an absorption maximum at a wavelength of 305 m μ . This absorption peak is distinct from the absorption spectra of the reacting substances and its magnitude is directly proportional to the amount of GSH present.

Other thiols, except γ -glutamyl cysteine, do not interfere with the GSH estimation unless present in large excess.

In the Patterson and Lazarow method GSH and Alloxan are mixed and the reaction initiated by bringing the pH to 7.5 by addition of sodium hydroxide and phosphate buffer.

However, it was found that after precipitation of protein with metaphosphoric acid addition of alkali and buffer to the supernatant did not always bring the reaction mixture to pH 7.5. As a result the estimation of GSH was erratic.

The procedure was modified so that the pH was brought to pH 7.5 before addition of the alloxan. This was done by titration with sodium hydroxide, using bromothymol blue as an indicator. An extra, predetermined amount of alkali, usually 0.1 ml., was added to compensate for the acidity of the alloxan added /

added later.

A strong acetate buffer, pH 7.5, was also used to ensure a pH of 7.5.

The reaction was initiated by the addition of the alloxan to the GSH solution at pH 7.5, and terminated by addition of excess alkali.

These modifications allowed reproducible results to be obtained.

Reagents:- 0.1 M Alloxan. 1.6 g. alloxan monohydrate dissolved in 100 ml. distilled water and stored in the deep freeze in 10 ml. aliquots. A fresh solution was made up at least once a week.

0.75 N Sodium Hydroxide.

1.0 M Acetate Buffer, pH 7.5. 136 g. $\text{CH}_3\text{COONa} \cdot 3\text{H}_2\text{O}$ dissolved in distilled water, made to pH 7.5 by the addition of glacial acetic acid, and the volume adjusted to 1 litre.

25% (w/v) Metaphosphoric acid.

1.0 N Sodium Hydroxide.

GSH Standard. 1 μM GSH per ml. made up in 5% metaphosphoric acid. Several dilutions of this standard were used to obtain the standard curve (Fig. M.8 and Table M.8).

Procedure:- /

Fig. M. 8

Standard Curve for Estimation of GSH
by the Modified Alloxan '305' Method

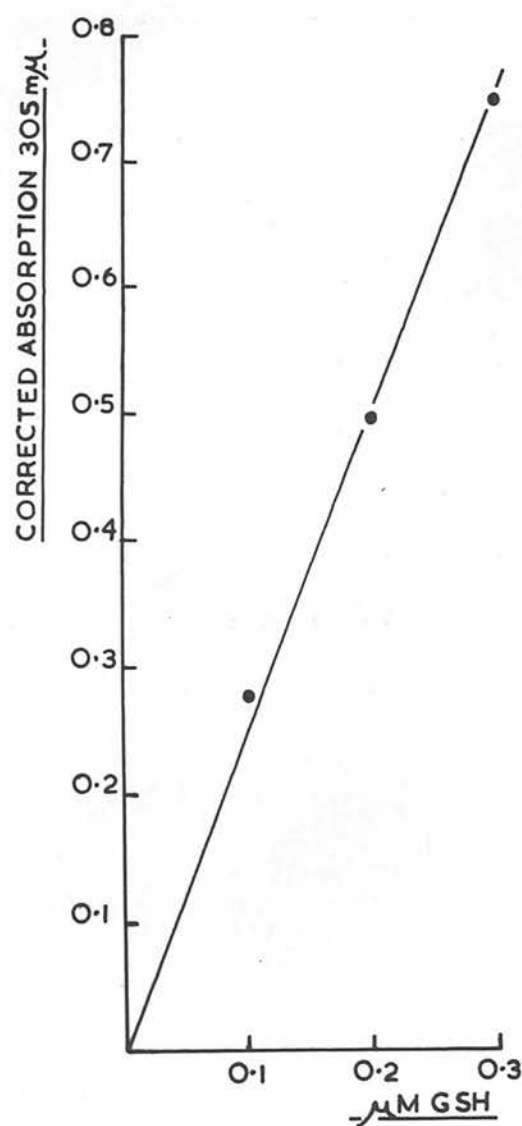


TABLE M. 8.

GSH STANDARDS FOR ALLOXAN '305' METHOD

| GSH Present μM | Optical density 305 mμ. | Mean | Mean minus blank |
|-------------------|----------------------------|-------|---------------------|
| 0.1 | 0.342 0.338 | 0.340 | 0.280 |
| 0.2 | 0.560 0.554 | 0.557 | 0.497 |
| 0.3 | 0.820 0.800 | 0.810 | 0.750 |
| Blank | 0.060 | 0.060 | 0 |

The Procedure:- To 1 ml. protein supernatant, or GSH standard, in 5% metaphosphoric acid was added 0.75 N NaOH to bring the solution to pH 7.5.

0.1 ml. 0.75 N NaOH was added after the end point, indicated by bromothymol blue, had been reached.

The volume was made up to 3 ml. with 1 M acetate buffer, pH 7.5, and the solutions placed in a water bath at 37°C.

Two identical solutions were prepared for each supernatant, one to act as the test solution and the other as a blank.

To the test solution was added 0.1 M alloxan solution (1 ml.) and to the blank distilled water (1 ml.).

After mixing the solutions remained in the water bath for exactly 6 minutes before 1 N NaOH (1 ml.) was added to both test and blank solutions.

All the solutions were brought to 37°C. before being used in the reaction.

The optical density of each test solution was then determined at 305 mμ, against the corresponding blank, in silica cuvettes of 1 cm. path length, and the GSH content determined from the standard curve (Fig. M.8.).

The Estimation of Non-Protein Disulphides.

Disulphides are most conveniently estimated after complete conversion to the corresponding thiol.

The reaction with sodium sulphite has proved to be the most generally useful, producing an equimolecular mixture of thiol and S-sulphocysteine derivatives, (Clarke, 1932).

Reduction of the disulphide by alkaline solutions of cyanide is the basis of the Sullivan and Hess procedure for the estimation of cystine, (Sullivan and Hess, 1937).

Methods of reduction which produce two moles of thiol per mole of disulphide have the advantage over the reactions with sulphite and cyanide but there are few satisfactory ways of reducing disulphides quantitatively.

Woodward and Fry (1932) used zinc dust and Schelling, (1932) used magnesium for the reduction of GSSG in acid solutions, while sodium amalgam in acid solution has brought about the reduction of CySSCy (Kolthoff and Stricks, 1950).

Perhaps the most promising method is that of electrolytic reduction introduced for GSSG by Dohan and Woodward, (1939). Reduction is carried out at a stirred mercury cathode.

This method has been used by Bhattacharya, Robson and Stewart, (1955) and Klebanoff (1957) for the determination of GSSG in blood.

However, /

However, this method is time consuming.

Enzymes exist which are capable of reducing disulphide compounds.

Glutathione reductase has occasionally been used to convert GSSG to GSH (Martin and McIlwain, 1959, and Mapson, (1953), the enzyme being supplied from an exogenous source.

Jocelyn, (1960) estimated GSSG in erythrocytes by using the reductase already present in the cells to reduce the disulphide.

The Estimation of GSSG.

It was found that when zinc was used to reduce GSSG in metaphosphoric acid the results were erratic and not reproducible.

When attempts were made to reduce GSSG present in the supernatants obtained by precipitating the proteins with metaphosphoric acid the recoveries were very low.

Other acids were tried but did not increase the extent of the reduction so attention was turned to the reduction of GSSG by glutathione reductase.

The method of enzymic reduction described by Jocelyn, (1960) was made the basis of the procedure.

The source of the glutathione reductase was freshly-drawn, oxalated /

oxalated blood.

Packed human erythrocytes were obtained by centrifuging the oxalated blood at 1000 g. for 10 minutes and removing the plasma and the buffy layer; the cells were washed once by suspension in twice their own volume of 0.9% sodium chloride (w/v) and recentrifuging.

The washed cells were lysed by the addition of 4 volumes of glass distilled water. This haemolysate, constituting the enzyme preparation, was used immediately.

Addition of the enzyme to a buffered system containing GSSG, NAD_1P , an oxidisable substrate such as glucose-6-phosphate (G-6-P) and sodium ethylene diamine tetra-acetate brought about complete reduction of GSSG in 30 minutes of 37°C . and pH 7.4.

Of the GSSG added to such a system 91 - 102% could be recovered as GSH, the mean recovery being 97.4% (Table M.9).

The GSH produced by the enzymic reduction was measured by the modified Alloxan '305' method (see p.52).

Enzymic Reduction

Reagents:- Glucose-6-phosphate. Solution
(1 μM /ml) made up in distilled water. Stored at 4°C .

NADP. Solution (1 μM /ml.) made up in distilled water immediately before use.

1.0 M. /

TABLE M. 9.

RECOVERY OF GSSG AS GSH FOLLOWING
ENZYMIC REDUCTION

0-1.0 μ M GSSG, 0.2 ml. 1:5 haemolysate,
0.1 μ M NADP, 1 μ M G-g-P, 2 μ M EDTA.
0.5 ml. 1M ACETATE BUFFER, pH 7.4
in total volume 4 ml. incubated at 37°C.
for 30 minutes. GSH estimated by
Alloxan '305' method after precipitation
of the proteins.

| <u>GSSG added</u> (μ M) | <u>GSSG recovered</u> (μ M) |
|---------------------------------|-------------------------------------|
| 0.08 | 0.08 |
| 0.16 | 0.16 |
| 0.32 | 0.29 |
| 0.40 | 0.41 |
| 0.48 | 0.45 |
| 0.60 | 0.60 |
| 0.80 | 0.76 |
| 1.00 | 0.92 |

1.0M. Acetate Buffer, pH 7.5 Stored at 4°C.

0.05 M EDTA. Used to chelate heavy metal ions. In its absence the recoveries of GSSG as GSH were erratic.

25% Metaphosphoric Acid (w/v).

0.75 N Sodium Hydroxide

GSSG Standards. 1 μ M GSSG per ml. Stored at 4°C.

Procedure:- To 2 ml. protein solution containing 1-2 μ M GSSG was added 25% metaphosphoric acid (0.5 ml.) and the protein precipitate removed by centrifugation.

1 ml. of the acid supernatant, or standard solution of GSSG in 5% metaphosphoric acid, was added to an equal volume of 0.75N sodium hydroxide and 1 M Acetate buffer, pH 7.5 (0.5 ml).

To the solution, at pH 7.5, 1.0 μ M G 6P, 0.1 μ M NADP, 2 μ M EDTA and 0.2 ml. of the 1 in 5 haemolysate were added and the volume made up to 4 ml.

The mixtures were then incubated for 30 minutes at 37°C. in a water bath, after which time the protein was precipitated by addition of 25% metaphosphoric acid (1 ml).

The GSH in the supernatant was estimated by the Alloxan '305' Method (p.52).

The amount of glutathione initially present in the haemolysate, which served as the source of the glutathione reductase, /

reductase, was always determined and subtracted from all the other values found when that particular enzyme preparation was used.

Thus a correction for the endogenous glutathione was applied.

Estimation of CySSCy.

The method used was that described by Sullivan and Hess, (1937).

This method has been used for the estimation of CySSCy and CySH following the acid hydrolysis of proteins. In the present work the CySSCy was present in the acid supernatants obtained by precipitating the protein from protein - CySSCy mixtures with metaphosphoric acid.

Reagents:- 5% (w/v) Sodium Cyanide in 1 N sodium hydroxide.

0.5% (w/v) aqueous sodium 1:2 naphtho-quinone -4-sulphonate made up immediately before use.

20% (w/v) sodium sulphite ($\text{Na}_2\text{SO}_3, 7\text{H}_2\text{O}$) in 0.5N sodium hydroxide.

5N sodium hydroxide.

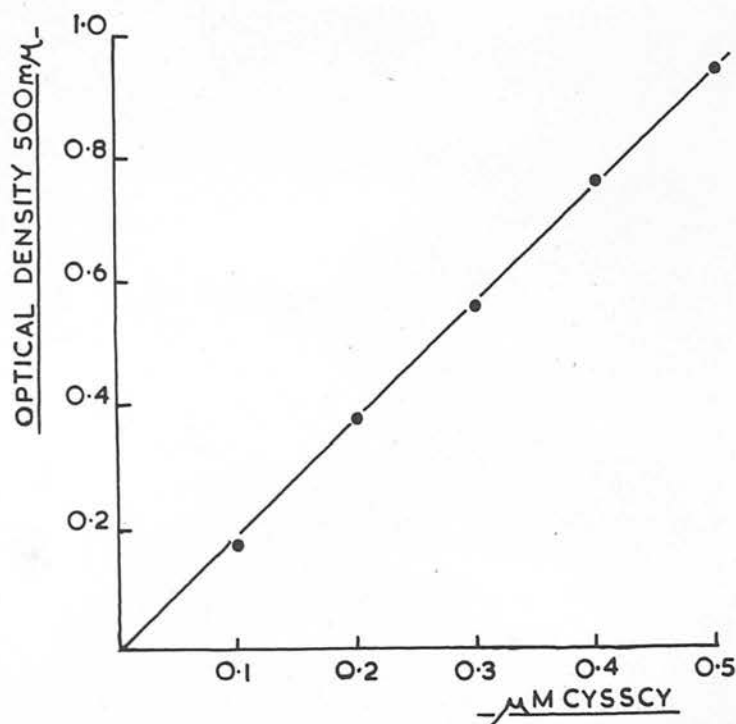
2% (w/v) sodium dithionite in 0.5N sodium hydroxide.

CySSCy standard. 0.25 μM per ml. in distilled water.

Procedure. /

Fig. M. 9

Standard Curve for CySSCy Estimation
by the Sullivan and Hess Procedure



Procedure. To 2 ml. metaphosphoric acid solution containing up to $0.5 \mu\text{M}$ CySSCy was added 5% NaCN (0.8 ml.), mixed well and stood for 10 minutes at room temperature ($22 - 24^{\circ}\text{C}$).

Naphthoquinone sulphonate reagent (0.4 ml) was then added and the mixture shaken for 10 seconds before the addition of 20% Na_2SO_3 (2 ml).

After mixing the reaction was allowed to proceed for 30 minutes and was then terminated by the addition of 5N NaOH (0.8 ml.) and 2% dithionite (0.4 ml.).

Total volume 6.4 ml.

The optical density of the red-brown solution was measured at 500 m μ , in cuvettes of 2 cm. light path, against a reagent blank 10 to 40 minutes after termination of the reaction.

The optical density always increased linearly with increase in CySSCy concentration (Fig. M.9) but the optical density was not always the same for a given amount of CySSCy, varying slightly from one experiment to another. Therefore standards were always included in each set of estimations.

CySSCy can be recovered from BSA - CySSCy mixtures to the extent shown in Table M.10; provided there is no delay in precipitation of the protein.

The Removal of Unreacted GSH and CySSCy from Reaction Mixtures.

TABLE M. 10.

To ensure the accurate determination of the protein

sulphydryl groups after incubation of haemoglobin with GSH

RECOVERY OF CySSCy FROM BSA-CySSCy MIXTURES

Several techniques were used in attempts to achieve this

0- 0.625 μ M CySSCy mixed with 0.5 μ M BSA
in total volume 4 mls. Protein precipitated
by addition of 1 ml. 25% metaphosphoric acid
CySSCy estimated in 2 ml. of supernatant.
Solutions with No. BSA treated identically.

the presence of the haemoglobin affected in some way, the

binding of GSH by the resin which was not effective in removing

GSH from haemoglobin-GSH mixtures.

| <u>CySSCy Solutions</u> <u>No. BSA</u> | | <u>CySSCy Solutions</u> <u>Containing BSA</u> | | <u>Recovery</u> % |
|---|---------------------------------|--|---------------------------------|----------------------|
| <u>Optical Density,</u> | <u>μM CySSCy</u> | <u>Optical Density,</u> | <u>μM CySSCy</u> | |
| 0.120 | 0.125 | 0.121 | 0.126 | 101 |
| 0.392 | 0.240 | 0.360 | 0.225 | 92 |
| 0.306 | 0.250 | 0.296 | 0.240 | 97 |
| 0.358 | 0.250 | 0.358 | 0.250 | 100 |

activity. (McCormack et al., 1969).

However, using this method complete removal of GSH

was difficult to achieve, a time interval of 18-20 hours being

required, although more than 80% of the GSH was removed in

4-5 hours

Over a week long periods of time the Hb lost up to 10% of

the

The Removal of Unreacted GSH and GSSG from Reaction Mixtures.

To ensure the accurate determination of the protein sulphydryl groups after incubation of haemoglobin with GSH it was necessary to remove, completely, the remaining GSH.

Several techniques were used in attempts to achieve this end speedily.

It was found that Dowex 50 resin in the hydrogen form could remove GSH from aqueous solutions very rapidly, but the presence of the haemoglobin affected, in some way, the binding of GSH by the resin which was not effective in removing GSH from haemoglobin-GSH mixtures.

Amberlite IRC 120 resin, which had been pretreated with pCMB, also bound GSH but, in the presence of haemoglobin, pCMB appeared to be released from the resin.

pCMB resins contained in dialysis sacs have been used to remove small molecular weight thiols essential for enzyme activity, (McCormack et al., 1960).

However, using this method complete removal of GSH was difficult to achieve, a time interval of 18-20 hours being required, although more than 80% of the GSH was removed in 4-6 hours.

Over such long periods of time the Hb lost up to 10% of its /

its sulphhydryl content, even in an atmosphere of nitrogen, at room temperature, (22-24°C).

These procedures were not as effective as the more lengthy procedure of dialysing the haemoglobin GSH mixtures against running water at 15-17°C for 22 hours.

During such lengthy dialysis little change occurred in the sulphhydryl content of the protein.

Consequently dialysis was adopted as the means of removing GSH, in the absence of a more speedy procedure.

GSSG did not interfere in the estimation of the protein sulphhydryl content by reaction with the pCMB but relatively high concentrations of GSSG used in some experiments did add considerably to the background absorption at 247 mμ.

It was found, however, that the GSSG could be removed, by Dowex 50, from mixtures of GSSG and protein without any effect upon the protein sulphhydryl content.

1 g. Dowex 50 removed 10 μM GSSG from 8 ml. solution within 15 mins.

INTRODUCTION

In the studies to be described the physiological occurrence of glutathione, glutathione (GSH) and cysteine (Cys), and their corresponding oxidized, disulfide glutathione (GS-SG) and cystine (Cys-S-Cys), were used in the investigation of possible reactions with sulphhydryl-containing proteins which occur in the blood.

The proteins studied were haemoglobin, which in vitro

RESULTS

exists in the erythrocytes along with a relatively high concentration of glutathione, and the plasma proteins, albumin and γ -globulin, which in vivo are in a medium containing only very small amounts of free protein disulfide (Hb-S-S-Hb) and GS-SG.

The aim of the investigation was to study any reaction between thiolate ion (SH-) and protein sulphhydryl groups under relatively mild conditions of temperature and pH, and where such a reaction was found to determine some of its characteristics.

INTRODUCTION

In the studies to be described the physiologically occurring thiols, glutathione (GSH) and cysteine (CySH), and their corresponding disulphides, oxidised glutathione (GSSG) and cystine (CySSCy), were used in the investigation of possible reactions with sulphhydryl containing proteins which occur in the blood.

The proteins studied were haemoglobin, which 'in vivo' exists in the erythrocytes along with a relatively high concentration of glutathione; and the plasma proteins, albumin and γ - globulin, which 'in vivo' are in a medium containing only very small amounts of non-protein thiols (NPSH) and disulphides (NPSS).

The aims of the investigation were to study any reaction between thiols and disulphides and protein sulphhydryl groups under relatively mild conditions of temperature and pH; and where such a reaction was found to determine some of its characteristics.

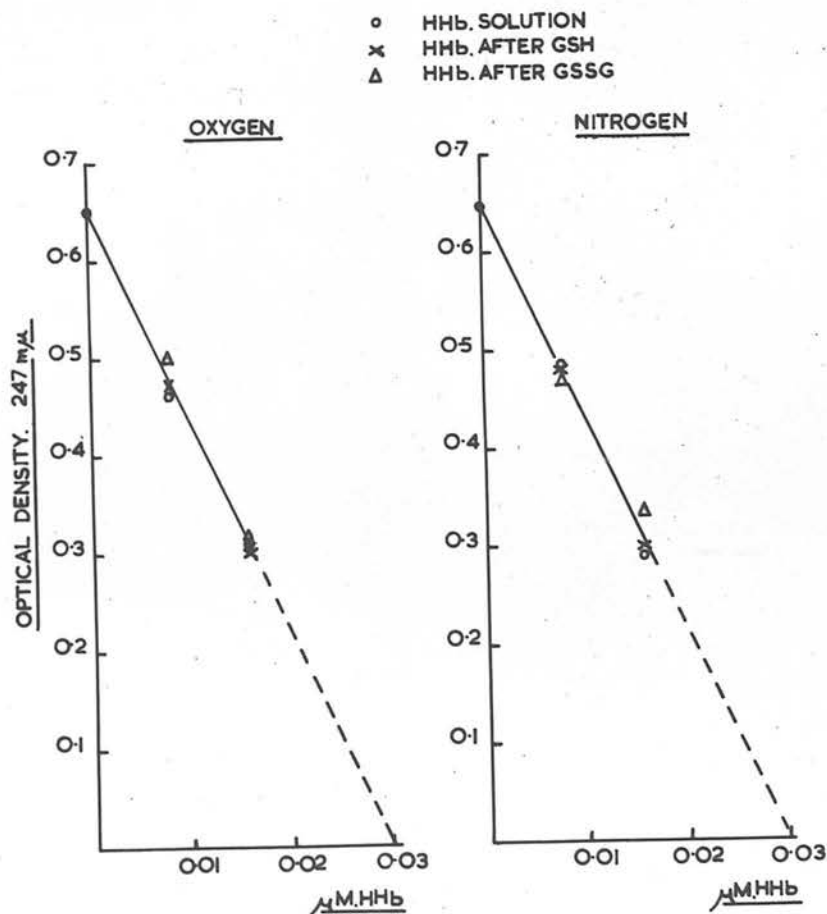
Fig. 1.

HHb with GSH or GSSG under Oxygen or Nitrogen

1.2 μM HHb incubated with 100 μM GSH or 50 μM GSSG in total volume 6 ml. Containing 0.2 mM phosphate buffer, pH 7.4, for 5 hours at 37°C.

Oxygen or Nitrogen, saturated with water vapour, bubbled through incubation mixtures at approximately 5 ml. per minute. Mixtures dialysed against running water (15-17°C) for 20 hours in 18/32" visking cellophane tubing.

Optical density measurements indicate the amount of pCMB removed on precipitation of HHb.



STUDIES WITH THE HAEMOGLOBINS

Human Haemoglobin (H Hb)

The incubation of GSH or GSSG with HHb in molecular ratios of 84:1 and 42:1 respectively, carried out either in oxygen or nitrogen, did not produce a significant reduction in the sulphhydryl content of the HHb.

Foaming of the haemoglobin solutions, which occurred on passing the gases, oxygen and nitrogen, through the solutions, was a problem in early experiments and led to a loss of protein sulphhydryl content. However, it was found that this could be controlled by the addition of 1 drop Silicone M.S. antifoam emulsion 'RD' (Hopkin & Williams Ltd., Chadwell Heath, Essex).

As can be seen from Fig. 1 the HHb treated with GSH or GSSG under either oxygen or nitrogen removed the same amount of pCMB from solution as did untreated HHb, indicating an identical sulphhydryl content.

In contrast to these results when the ratio of GSH or GSSG to HHb was increased, to 132:1 or 66:1 respectively, the results shown in Table 1 were obtained.

Incubation for 5 hours caused no loss of sulphhydryl content in the HHb solutions not treated with thiol or disulphide, but prolonged dialysis in this case brought about a loss /

TABLE 1.

0.75 μ M HbA incubated with 100 μ M GSH or 50 μ M GSSG for 5 hours at 37°C. pH 7.4. Total volume 6 mls, including 0.2 mM phosphate buffer pH 7.4. Dialysed 20 hours against running water after incubation.

Sulphydryl Content
(μ M SH / μ M HbA)

| | <u>Oxygen</u> | | <u>Nitrogen</u> | |
|--------------|--------------------|-------------------|--------------------|-------------------|
| | Before Dialysis | After Dialysis | Before Dialysis | After Dialysis |
| Hb A control | 6.1 | 5.1 | 6.0 | 5.2 |
| HbA + GSH | - | 3.2 | - | 4.6 |
| HbA + GSSG | - | 4.8 | - | 4.8 |

loss of approximately one sixth of the sulphhydryl content.

From Table 1. it can be seen that GSH, in the presence of oxygen; caused a considerable decrease in the HHb sulphhydryl content. Such a large decrease did not occur in the solutions incubated under nitrogen or treated with GSSG in oxygen.

The high GSH to HHb ratio required to produce a change in the protein sulphhydryl content can be contrasted with the situation existing 'in vivo' where the GSH to HHb molar ratio is of the order of 1:2.

The treatment of the HHb with GSH in the presence of oxygen was accompanied by the formation of a very insoluble precipitate devoid of sulphhydryl groups. This kind of precipitate was not formed to any appreciable extent in the other incubation mixtures.

High thiol concentrations are known to bring about the dissociation of the peptide chains of HHb. (Cecil and McPhee, 1959). Such dissociation could lead to the exposure of sulphhydryl groups, which could react with the GSSG, present in the oxidising conditions, to form a mixed-disulphide, or the sulphhydryl groups could be oxidised by molecular oxygen. In either case removal of the sulphhydryl groups might be followed by precipitation.

TABLE 2

EFFECT OF INCUBATION OF GSSG WITH
BOVINE HAEMOGLOBIN

0.22 μ M Hb incubated with 0-10 μ M GSSG in a total volume of 6 ml. Buffered by 0.2mM phosphate buffer pH 7.4. Incubation at 37°C N₂ SH content by pCMB method, triplicate determinations.

| μ M GSSG present initially | Sulphydryl Content μ M SH/ μ M Hb |
|-----------------------------------|--|
| 0 | 1.8 |
| 2.5 | 2.0 |
| 5.0 | 1.9 |
| 7.5 | 2.0 |
| 10.0 | 1.8 |

4. Haemoglobin and GSH. In a medium buffered to

pH 7.4, 1 μ M of haemoglobin was incubated for 5 hours at 37°C

with GSH in concentrations ranging from 0-150 μ M and through

oxygen saturation was bubbled.

After washing with water (4-17°C) followed and the

SH content of the haemoglobin in the GSH solution

was determined.

The results shown in Table 2 indicate that

Allen and Jandl, (1961) found that the blockage of more than two sulphhydryl groups by pCMB in the presence of oxygen accelerated the precipitation of HHb.

These authors using ^{35}S -labelled GSH also reported the binding of a small amount of the GSH to HHb probably through a mixed disulphide bond.

Bovine Adult Haemoglobin.

1. Haemoglobin and GSSG. No appreciable change in sulphhydryl content of the bovine haemoglobin was found following incubation of the protein with varying amounts of GSSG up to 50 moles GSSG per mole of haemoglobin.

The incubation was carried out at 37°C and pH 7.4 for 15 hours under nitrogen and the values obtained for the sulphhydryl content of the haemoglobin in the incubation mixtures after this treatment are shown in Table 2.

2. Haemoglobin and GSH. In a medium buffered to pH 7.4, $1\text{ }\mu\text{M}$ of haemoglobin was incubated for 5 hours at 37°C with GSH in concentrations ranging from 0-150 μM and through these mixtures oxygen was bubbled.

Dialysis against running water ($15-17^{\circ}\text{C}$) followed and the sulphhydryl content of the haemoglobin in the GSH-free solutions was then determined.

The results shown in Table 3. indicated a considerable loss /

TABLE 3.

EFFECT OF GSH UPON THE SULPHYDRYL CONTENT
OF BOVINE HAEMOGLOBIN

1 μ M Hb incubated at 37°C and pH 7.4 for 5 hours in presence of O_2 with 0-150 μ M GSH. Total volume 6 mls. containing 0.2 mM phosphate. Buffer pH 7.4. Dialysed 20 hrs. against running water. SH content by pCMB method.

| | μ M GSH Present Initially | | | | |
|---|-------------------------------|-----|-----|-----|-----|
| | 0 | 50 | 100 | 125 | 150 |
| Sulphydryl content μ M SH/ μ M Hb. | 1.9 | 2.2 | 2.0 | 2.0 | 0.9 |

TABLE 4.

INSOLUBLE RESIDUE FORMED BY ACTION OF GSH
ON BOVINE HAEMOGLOBIN

Conditions of reaction as in Table 3.

| μ M GSH present initially per μ M Hb. | 0 | 100 | 125 | 150 |
|--|------|------|------|------|
| Wt. of Hb in Aliquot initially (mg.) | 45.2 | 45.2 | 45.2 | 45.2 |
| Wt. of dried residue. (mg.) | 1.1 | 1.9 | 4.4 | 17.5 |

loss of titratable sulphhydryl groups where the haemoglobin had been treated with 150 μ M GSH.

As had been found with human haemoglobin the loss in sulphhydryl content was accompanied by the formation of a finely divided but extremely insoluble precipitate.

An aliquot of each incubation mixture yielded some precipitate, which after washing three times with distilled water was dried to constant weight in a vacuum desiccator over calcium chloride.

Only in the case where the haemoglobin was treated with 150 μ M GSH per μ M haemoglobin was there an appreciable amount of the very insoluble precipitate formed. (Table 4.)

Further experiments were designed to determine the effect the length of incubation had upon the reaction of GSH with bovine haemoglobin.

Periods of incubation from 0 to 4 hours were tried with GSH and haemoglobin present in the molar ratio of 150 to 1 and as Table 5. shows quite clearly no change occurred in the sulphhydryl content of the haemoglobin on incubation and dialysis unless both oxygen and GSH were present.

The maximum effect was obtained within an incubation time of one hour.

At /

TABLE 5.

EFFECT OF LENGTH OF INCUBATION ON REACTION
BETWEEN GSH AND BOVINE HAEMOGLOBIN

2.5 μM Hb with or without 375 μM GSH incubated at 37°C and pH 7.4. In the presence of oxygen. Total volume 15 mls. 2 ml. aliquots taken at hourly intervals, dialysed 20 hours. Sulphydryl determined by pCMB method.

| | Control Hb (No GSH) | | Hb + GSH (150 μM GSH/ μM Hb) | | | | |
|--|------------------------|-----|---|-----|-----|-----|-----|
| Time incubated (hours) | 0 * | 4 | 0* | 1 | 2 | 3 | 4 |
| Sulphydryl content μM SH/ μM Hb | 1.9 | 2.2 | 2.0 | 0.9 | 0.9 | 1.0 | 0.8 |

* oxygen not passed through these solutions

At this stage this aspect of the problem was left, the precipitate being so insoluble as to prevent adequate study; and because of the extremely large amounts of GSH required to bring about the effect it would not have been practicable to try to estimate the amount of GSH removed, if any, in the formation of some chemical linkage, such as a mixed disulphide bond.

At the most this would have accounted for 2 μM GSH out of a total of 150 μM . Losses due to oxidation would undoubtedly have been much greater under the conditions of the experiments.

On the whole it appears that neither human nor bovine haemoglobins reacted with GSSG to any measurable extent.

The effect found with both the haemoglobins when treated with large amounts of GSH in the presence of oxygen could conceivably be due to the dissociation of the peptide chains of the protein in the high concentration of thiol.

The oxygen present might then have oxidised the exposed sulphydryl groups, so preventing a reversal of the dissociation on removal of the excess GSH, and eventually leading to precipitation of the protein.

No evidence for the formation of mixed disulphide between either haemoglobin and GSSG was found under the conditions employed.

3. Haemoglobin and CySSCy. The addition of a solution of CySSCy to a solution of haemoglobin buffered to pH 7.4 and at /

at a temperature of 37°C produced a decrease in the sulphydryl content of the protein within 20 hours. (Table 6a.)

With increasing CySSCy concentration the amount of protein sulphydryl removed increased up to 1 μ M sulphydryl per μ M haemoglobin.

On account of the low solubility of CySSCy it was not feasible to increase its concentration in the medium, so the concentration of haemoglobin was reduced by half in further experiments, thereby doubling the molecular ratio of CySSCy to haemoglobin.

Again a maximum of one sulphydryl group per molecule of haemoglobin was lost. (Table 6b.)

From the results obtained it was quite clear that CySSCy reacted with haemoglobin bringing about a decrease in the sulphydryl content of the protein.

Under the conditions employed here this decrease was never more than one sulphydryl group per haemoglobin molecule.

TABLE 6.

EFFECT OF CYSTINE UPON SULPHYDRYL CONTENT
OF BOVINE HAEMOGLOBIN

A. 1.2 μM Hb incubated at 37°C and pH 7.4 for 24 hours with 0-0.75 μM CySSCy. Total volume 4 mls. containing 0.5 mM Tris buffer pH 7.4. SH content by pCMB method.

| | | | | |
|--|-----|------|------|------|
| μM CySSCy per μM Hb present initially | 0 | 0.21 | 0.42 | 0.63 |
| μM SH per μM Hb after 20 hours incubation. | 2.1 | 1.7 | 1.3 | 1.1 |

B. 0.6 μM Hb with 0.0.75 μM CySSCy incubated as in Table 6A.

| | | | | |
|---|-----|------|------|------|
| μM CySSCy per μM Hb present initially | 0 | 0.84 | 1.10 | 1.25 |
| μM SH per μM Hb after incubation. | 2.0 | 1.2 | 1.2 | 1.0 |

Bovine- γ - Globulin and GSSG

The preparation of bovine γ -globulin used in these studies had a fractional sulphhydryl content per molecule as indicated earlier in the description of the PCMB method for sulphhydryl estimation.

This made it rather unsuitable for use with the pCMB method as a protein solution of high concentration was required to achieve the removal of a suitable amount of pCMB. The removal of pCMB led to a decrease in the optical density of the protein free supernatant at 247 m μ and this decrease was therefore a measure of the protein sulphhydryl content. Obviously the larger the change in absorption the more accurate the assessment of the sulphhydryl content tended to be.

However, attempts were made with less concentrated solutions to study the possible effects of GSSG upon the sulphhydryl content of the bovine- γ - globulin.

The small variations found after GSSG treatment were not considered to be significant or indicative of a general reaction between GSSG and the protein sulphhydryl group. (Table 7.).

TABLE 7.

EFFECT OF GSSG UPON SULPHYDRYL CONTENT OF
BOVINE - γ - GLOBULIN

0.5 μ M bovine- γ - globulin incubated at 37°C and pH 7.4 for 15 hours with 0-10 μ M GSSG in total volume 8 mls. containing 0.2 mM phosphate buffer pH 7.4. pCMB method for sulphydryl content.

| μ M GSSG per μ M globulin Present initially | 0 | 5.0 | 10 | 15 | 20 |
|--|------|------|------|------|------|
| μ M SH per μ M globulin After incubation. | 0.29 | 0.23 | 0.29 | 0.28 | 0.26 |

TABLE 8.

BOVINE SERUM ALBUMIN AND GSSG

1 μ M BSA incubated with 0-5 μ M GSSG at 37°C, pH 7.4 for 1 hour. Total volume 8 mls. Containing 0.4 mM phosphate buffer pH 7.4. Sulphydryl content by pCMB method. GSSG by Alloxan '305' method after enzymic reduction.

| μ M GSSG initially present. | 0 | 1.0 | 2.0 | 3.0 | 4.0 | 5.0 |
|--|------|------|------|------|------|------|
| μ M SH per μ M BSA after incubation. | 0.66 | 0.65 | 0.64 | 0.64 | 0.66 | 0.66 |
| μ M GSSG recovered after incubation. | 0 | 1.14 | 2.14 | 2.76 | 4.26 | 5.14 |

Reactions of Serum Albumin with Thiols and Disulphides.

Bovine serum albumin (BSA) and human serum albumin (HSA) both have a sulphydryl content of 0.65 sulphydryl groups per molecule. (see pages 38, 39).

The sulphydryl group is stable and not readily oxidised by atmospheric oxygen during prolonged incubation.

The intact proteins give only a faint nitroprusside test but the sulphydryl groups react readily with pCMB, a point of convenience in the estimation of the sulphydryl content.

Bovine Serum Albumin and GSSG. As with the other sulphydryl proteins studied bovine serum albumin did not suffer a loss of sulphydryl content on incubation with GSSG.

In the first instance the length of incubation was limited to one hour with 0 to 5 moles GSSG per mole of albumin. This produced no reduction in the sulphydryl content of the protein nor was there any loss of GSSG from the medium. (Table 8.).

Failure to obtain any evidence of an interaction between GSSG and BSA led to the use of a wider range of GSSG concentrations and a lengthier incubation period.

To prevent possible oxidation of the protein sulphydryl group the incubation was carried out in Thunberg tubes which were evacuated and then filled with nitrogen.

Even with the longer incubation and the larger amounts of /

TABLE 9.

BSA WITH LARGER AMOUNTS OF GSSG

Conditions as in Table 8. but 0-20 μ M

GSSG present initially.

Incubation prolonged for 15 hours.

Sulphydryl by pCMB method

Mean of triplicates.

| <u>μM GSSG</u> <u>present initially</u> | <u>μM SH per μM BSA</u> <u>after incubation</u> |
|---|--|
| 0 | 0.66 |
| 1.0 | 0.66 |
| 2.0 | 0.68 |
| 3.0 | 0.69 |
| 4.0 | 0.67 |
| 5.0 | 0.63 |
| 7.5 | 0.63 |
| 10.0 | 0.63 |
| 15.0 | 0.65 |
| 20.0 | 0.66 |

of GSSG no decrease in the sulphydryl content was found which was significant. (Table 9.)

The amounts of GSSG remaining in the medium were also checked after the 15 hours incubation and compared with controls which had not been incubated but from which the BSA had been precipitated immediately after mixing the GSSG and protein. Standard solutions of GSSG incubated for 15 hours were also analysed for their GSSG content after incubation.

Some variation was found between the results of different experiments and Table 10. shows the mean findings of seven identical experiments.

It was found that on incubation for 15 hours some GSSG was lost from all the solutions whether albumin was present or not. Smaller losses of GSSG were also found to occur when the albumin was precipitated immediately after mixing of the BSA and GSSG. The losses in this latter case were of the same order as the differences between the amounts of GSSG recovered from incubation mixtures, which had contained albumin, and from the incubated standard GSSG solutions, which had not.

Thus it appeared that no GSSG was removed from the incubation mixtures by reaction with BSA.

Attempts were also made to demonstrate the formation of GSH during the incubation of GSSG with BSA.

After /

TABLE 10

GSSG REMAINING AFTER INCUBATION WITH BSA

0.5 μ M incubated with 0-5 μ M GSSG at 37°C,
pH 7.4 for 15 hours. Total volume 4 ml.
containing 0.2 mM phosphate buffer pH 7.4
GSSG estimated in protein-free supernatants
by Alloxan '305' after enzymic reduction.

| μ M GSSG initially present | μ M GSSG Recovered | | |
|-----------------------------------|-----------------------------------|------------------------------|---|
| | Incubated BSA present \pm SD | Incubated No BSA \pm SD | Not incubated. BSA immediately precipitated \pm SD |
| 0 | 0 | 0 | 0 |
| 1.25 | 1.08 \pm 0.09 | 1.08 \pm 0.13 | 0.38 |
| 2.50 | 2.06 \pm 0.16 | 2.24 \pm 0.24 | 2.37 |
| 3.75 | 2.81 \pm 0.47 | 3.16 \pm 0.38 | 3.28 |
| 5.00 | 4.14 \pm 0.21 | 4.23 \pm 0.33 | 4.90 |

After incubation of the reactants for 15 hours both the Alloxan '305' and the pCMB methods, described in the methods section, were used to detect any GSH formed. These estimations were carried out on solutions from which the protein had been precipitated with metaphosphoric acid. (Table 11.)

No significant increase in optical density at 255 m μ was found on addition of pCMB, indicating the absence of GSH from the acid supernatants, while with the Alloxan '305' method the amounts of "GSH" detected were greatest in the solutions which had not contained GSSG. (Table 11.)

These indicated amounts of GSH were at the very limit of sensitivity of the Alloxan '305' method and probably did not represent the presence of real GSH.

Thus it was concluded that if any GSH was produced and had survived reoxidation it was such a small amount that it could not be detected by the available methods.

Catalysis by Copper Ions. Ions of heavy metals facilitate the oxidation of thiols to disulphides in the presence of oxidising agents such as oxygen and hydrogen peroxide.

Oxidation of the sulphydryl form of BSA to form a disulphide dimer is only brought about under special conditions, (Straessle, 1954) but it was considered possible that the ions of heavy metals could catalyse a reaction in the presence of the /

TABLE 11.

GSH PRODUCED

0.5 μ M incubated with 0-5 μ M GSSG at 37°C and pH 7.4.

Total volume 4 mls.

After incubation protein precipitated. Aliquots of supernatant for Alloxan '305' and pCMB methods.

| μ M GSSG present initially | Optical density at 255 m μ (pCMB method) | μ M 'GSH' found (Alloxan '305' method) |
|-----------------------------------|--|--|
| 0 | 0.248 | 0.10 |
| 0.25 | 0.240 | 0.10 |
| 2.50 | 0.241 | 0.05 |
| 3.75 | 0.248 | 0.08 |
| 5.00 | 0.256 | 0.03 |

Fig. 2.

Catalytic Effect of Copper Ions on Interaction
of GSSG and BSA

0.5 μ M BSA incubated with 0, 2.5 and 5 μ M GSSG for 15 hours at 37°C and pH 7.4 under N₂. Total volume 4 ml. containing 20 μ g copper. Also controls with no copper. SH content by pCMB method.

The extinctions of all solutions at 247 m μ were within the limits of the vertical lines.

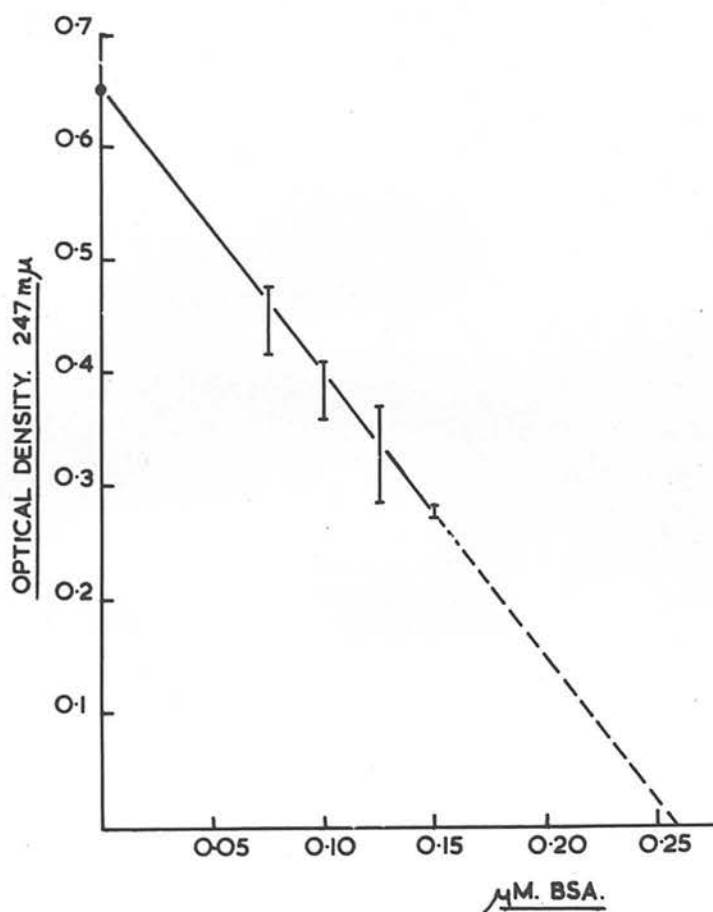


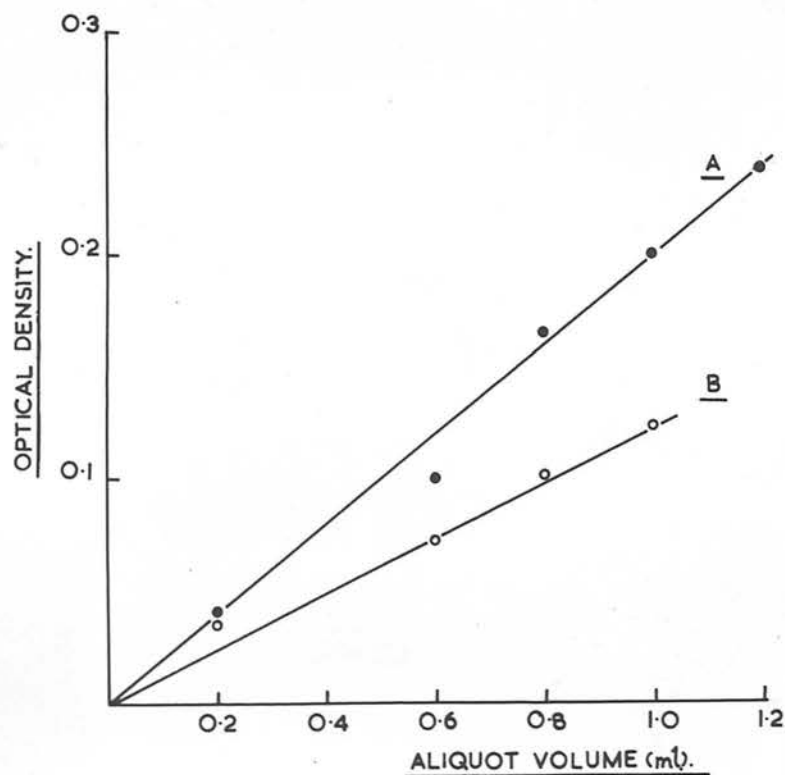
Fig. 3.

Effect of Cupric Ions on the Absorption of GSSG
at 247 m μ

2.5 μ M GSSG with or without 20 μ gCu⁺⁺ as CuSO₄,
in total volume 4 ml.

Aliquots diluted to 3 ml. optical densities
measured at 247 m μ .

- A. GSSG solution with 20 μ gCu⁺⁺
B. GSSG solution. No Cu⁺⁺



the mild oxidising agent, GSSG, to bring about the formation of a mixed disulphide. The reaction was carried out in an atmosphere of nitrogen.

The only catalysts as yet known for thiol-disulphide interchanges are other sulphydryl compounds.

The incubation of BSA with up to 10 moles GSSG per mole protein, with or without catalytic amounts of copper, as cupric sulphate, did not bring about any change in the protein sulphydryl content. This was shown by the removal of an equivalent amount of pCMB from solution by equal aliquots of the various incubation mixtures (Fig. 2.)

The extinctions at 247 m μ indicated a mean sulphydryl content of 0.62 moles sulphydryl per mole of BSA.

It is possible, however, that the cupric ions were not available to act as catalysts.

Klotz et al., (1955) showed that BSA could bind cupric ions and it was found here that addition of the metal ions to a solution of GSSG resulted in an increase in absorption at 247 m μ suggesting the formation of cupric ion - GSSG complex. (Fig. 3.)

Nothing is known about the ability of ions bound in this way to act as catalysts.

Human Serum Albumin (HSA) and GSSG. The properties of HSA and BSA are very similar and they have identical sulphydryl /

TABLE 12

INCUBATION OF HSA WITH GSSG

0.5 μ M HSA incubated with 0-5.0 μ M GSSG for 15 hours under N_2 at $37^\circ C$ and pH 7.4. Total volume 4 mls. containing 0.2 mM phosphate buffer pH 7.4. Sulphydryl content by pCMB method. GSSG by Alloxan '305' method after enzymic reduction.

A. Sulphydryl Content

| | | | | | |
|---|------|------|------|------|------|
| μ M GSSG Present initially | 0 | 1.25 | 2.50 | 3.75 | 5.00 |
| μ M SH per μ M HSA after incubation | 0.64 | 0.63 | 0.63 | 0.64 | 0.60 |

B. GSSG content of medium after incubation

| | | | | | |
|---|---|------|------|------|------|
| μ M GSSG Present initially | 0 | 1.25 | 2.50 | 3.75 | 5.00 |
| μ M GSSG recovered Incubated with HSA | 0 | 1.17 | 2.13 | 3.25 | 4.50 |
| μ M GSSG recovered Incubated No HSA | 0 | 1.25 | 2.25 | 3.75 | 4.75 |
| μ M GSSG Recovered No incubation. HSA immediately precipitated. | 0 | 1.25 | 2.37 | 3.28 | 4.90 |

sulphydryl contents.

Experiments carried out under the same conditions as those used for BSA and GSSG did not indicate that there was any difference between the sulphydryl group of BSA and that of HSA in so far as reaction with GSSG was concerned.

No change in sulphydryl content of HSA was found, nor was there any significant loss of GSSG from the incubation medium, which could be said to have been due to interaction of the disulphide with the protein. (Table 12a - b).

The largest fall in the sulphydryl content was approximately 6%, while at the most the amount of GSSG lost on incubation with HSA was 3% of the original.

Thus it appeared that, as with BSA, there was no reaction of the protein sulphydryl group with the non-protein disulphide under the conditions of these experiments.

Reaction of Bovine Serum Albumin with Cystine. Having failed to find any evidence for interaction between BSA, or HSA, and GSSG, attention was turned to the non-protein disulphide of smaller molecular weight, cystine.

This amino acid consists of two molecules of cysteine linked through a disulphide bond. This molecular arrangement is responsible for many of the spatial arrangements of proteins and /

TABLE 13.

EFFECT OF CySSCy ON BSA SULPHYDRYL CONTENT

1 μ M BSA incubated with 1 μ M CySSCy at 37°C, pH 7.4 for 20 hours. Total volume 8 ml. containing 1.6 mM Tris-HCl. Buffer, pH 7.4 Incubation in air or evacuated Thunberg Tubes. Sulphydryl content by the pCMB method. Dialysis was for 19 hours against distilled water at 4°C in 18/32 in. Visking cellophane tubing.

| | | Sulphydryl Content μ MSH per μ M BSA | |
|------------|------------------|---|----------|
| | | In air | In vacuo |
| Undialysed | BSA No CySSCy | 0.69 | 0.69 |
| | BSA + CySSCy | 0.19 | 0.13 |
| Dialysed | BSA No CySSCy | 0.69 | 0.69 |
| | BSA + CySSCy | 0.17 | 0.14 |

and is also found in GSSG.

A somewhat unfortunate property of cystine is its low solubility in aqueous solutions. This is in direct contrast to GSSG which is very soluble in water.

As a preliminary step in the study of the reaction of cystine with BSA attempts were made to demonstrate a reduction in the sulphhydryl content of the protein on incubation with CySSCy.

Table 13. shows that a decrease of 75% in the titratable sulphhydryl was achieved with cystine and that the decrease was slightly greater in the absence of air.

Dialysis of the solutions for 19 hours after incubation did not alter, appreciably, the sulphhydryl content, indicating that the reaction was not easily reversible and that, probably, there was no CySH present after the incubation.

Cysteine, a possible product of the protein sulphhydryl-CySSCy interaction, is, however, very readily oxidised in alkaline media.

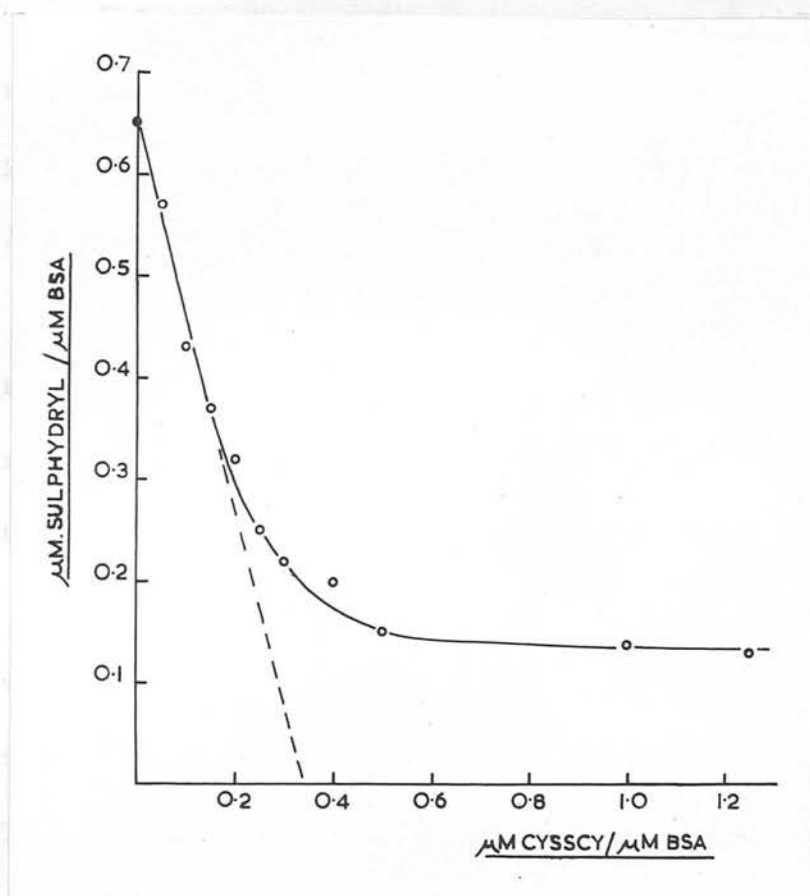
When the BSA was reacted with increasing amounts of CySSCy the sulphhydryl content fell sharply at first until a ratio of approximately 0.5 moles CySSCy per mole of BSA was reached, beyond which point there was little change in the sulphhydryl content with further increase in the cystine concentration. (Fig. 4).

Fig. 4.

BSA Sulphydryl Content and Increasing Amounts of CySSCy

1 μM BSA incubated with increasing amounts of CySSCy at 37°C , pH 7.4 for 20 hours. Total volume 8 ml.

Sulphydryl estimated by pCMB method.



Over the first portion of the curve (Fig. 4) the sulphydryl content decreased linearly, with increase in CySSCy, by an amount equivalent to twice the amount of CySSCy added i. e. equivalent in fact to the $\frac{1}{2}$ CySSCy concentration.

Extrapolation of this linear portion of the curve to the abscissa indicated that complete removal of the BSA sulphydryl group would have been attained with a BSA - CySSCy ratio of 1 to 3.4 if the initial reaction had been maintained.

To obtain a more complete picture of the disulphide-sulphydryl interaction, cystine remaining in the incubation mixtures at the termination of the reaction was estimated by the Sullivan and Hess procedure. (see methods).

Thus when a constant amount of cystine was incubated, with increasing amounts of BSA, the CySSCy content of protein-free aliquots of the reaction mixtures could be determined. The results listed in Table 14. were obtained.

The mean value for the amount of CySSCy removed from the reaction mixture by BSA, which had a sulphydryl content of $0.65 \mu\text{M SH per } \mu\text{M BSA}$, was $0.33 \mu\text{M CySSCy per } \mu\text{M BSA}$.

The two types of experiment indicated that 0.65 molecules BSA; or more probably one molecule of bovine mercaptalbumin, combined with one $\frac{1}{2}$ CySSCy molecule.

Possible /

TABLE 14.

CySSCy REMOVAL WITH INCREASING
AMOUNTS OF BSA

0.50 μ M CySSCy incubated with increasing amounts of BSA at pH 7.4 and 37°C. Total volume 4 ml.

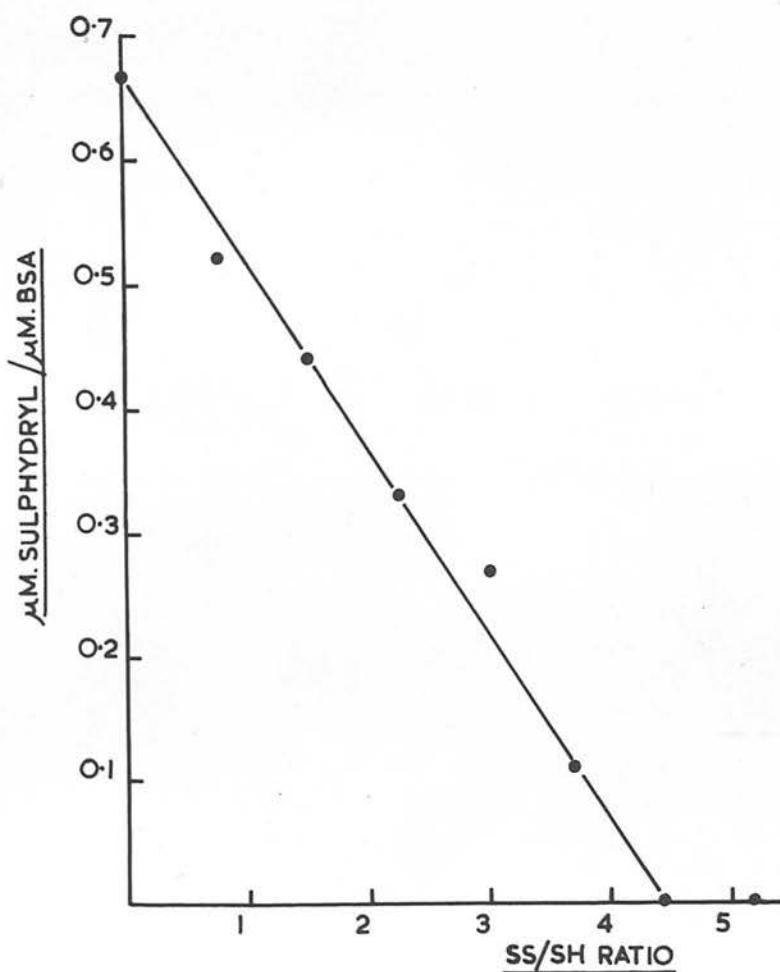
CySSCy estimated in 2 ml aliquots by Sullivan and Hess method.

| | | | | | | | |
|--|-----|------|------|------|------|------|------|
| μ M BSA present | 0 | 0.1 | 0.2 | 0.3 | 0.4 | 0.5 | 0.6 |
| μ M CySSCy present initially. | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 |
| μ M CySSCy after incubation. | 0.5 | 0.46 | 0.44 | 0.40 | 0.37 | 0.33 | 0.32 |
| μ M CySSCy removed per μ M BSA | - | 0.40 | 0.30 | 0.33 | 0.33 | 0.34 | 0.30 |
| Mean value | | | | 0.33 | | | |
| SH content μ M SH per μ M BSA | | | | 0.65 | | | |

Fig. 5.

Complete Removal of BSA Sulphhydryl by Cystine

0.5 μ M BSA (0.33 μ M mercaptalbumin) incubated at 37°C, pH 7.4 for 20 hours with 0-1.75 μ M CySSCy. Total volume 4 ml. Sulphydryl content by pCMB method.



Possible products of the interaction of cystine and BSA were, a protein disulphide dimer, a mixed disulphide dimer and cysteine.

Attempts to detect any cysteine produced by incubation of cystine with BSA were unsuccessful, even when the reaction was carried out in evacuated Thunberg tubes.

However, small amounts of a standard solution of CySH incubated overnight under the same conditions were not detectable by the available methods and no doubt this was due to the ease of oxidation of CySH, especially in an alkaline medium.

As it had not been possible to bring about a complete removal of the BSA sulphydryl group by treatment with cystine under the conditions of the previous experiments the albumin concentration was reduced by half.

This allowed a wider range of albumin SH to cystine SS ratios to be achieved.

Under the new conditions of reaction it was possible to bring about a complete removal of titratable sulphydryl group from the protein (Fig. 5).

Effect of p-Chloromercuribenzoate on the Reaction. As it has been shown that the sulphydryl content of BSA diminishes on reaction with CySSCy and that at low cystine concentrations the /

TABLE 15

EFFECT OF pCMB

Equivalent amounts of pCMB and BSA reacted at room temperature for 10 minutes, then dialysed overnight at 4°C against glass distilled water. 1 μ M pCMB-treated BSA incubated with 1 μ M CySSCy at 37°C. and pH 7.4 for 20 hours in total volume 8 mls. Sulphydryl content and cystine content estimated before and after incubation.

| | Before Incubation | | After Incubation | |
|------------------------------|--|------------------------------|--|------------------------------|
| | SH content (μ M SH/ μ M BSA) | CySSCy content (μ M) | SH content (μ M SH/ μ M BSA) | CySSCy content (μ M) |
| pCMB treated BSA + CySSCy | 0.08 | 0.504 | 0.09 | 0.496 |
| Untreated BSA + CySSCy | 0.64 | 0.488 | 0.12 | 0.240 |
| Untreated BSA No CySSCy | 0.67 | - | 0.66 | 0. |

the BSA and CySSCy reacted in the proportion of one BSA sulphhydryl to one $\frac{1}{2}$ CySSCy, the effect of pCMB upon the binding of cystine was investigated.

Sodium pchloromercuribenzoate reacts rapidly with sulphhydryl groups and unless it is present in excessive amounts it does not react with other groups within the protein molecule.

Equivalent amounts of BSA and pCMB were mixed, stood for 10 minutes and then dialysed overnight at 4°C against glass distilled water to remove excess unreacted mercurial.

Following this treatment, which removed the titratable sulphhydryl of the BSA, the dialysed protein along with untreated protein as a control, was incubated with cystine.

Analyses for sulphhydryl content and the cystine content of the media were carried out before and immediately after the incubation. (Table 15).

It can be clearly seen that pCMB treatment of the albumin, prior to the reaction with cystine, prevented any removal of cystine from the reaction medium. In the untreated albumin solution, reacted with cystine, the sulphhydryl content dropped by 0.52 μM SH per μM BSA- with a concomitant drop in the cystine concentration of the medium equivalent to 0.496 μM $\frac{1}{2}$ CySSCy.

These findings make it quite evident that the sulphhydryl group /

TABLE 16.

REACTION OF BSA AND CySSCy.
TIME EFFECT

BSA and CySSCy, in molar ratio of 2:1, incubated at 37°C and pH 7.4 in 0.2 M tris-Hcl Buffer.
Sulphydryl content by pCMB method.
CySSCy by Sullivan and Hess procedure.

| Time (hours) | SH content (μM SH/μM BSA) | $\frac{1}{2}$ CySSCy concentration per μM BSA |
|--------------|---------------------------|---|
| 0 | 0.65 | 0.900 |
| 4 | 0.40 | 0.592 |
| 6 | 0.34 | 0.520 |
| 8 | 0.26 | 0.492 |
| 10 | 0.25 | 0.460 |
| 24 | 0.21 | 0.388 |

Fig. 6.

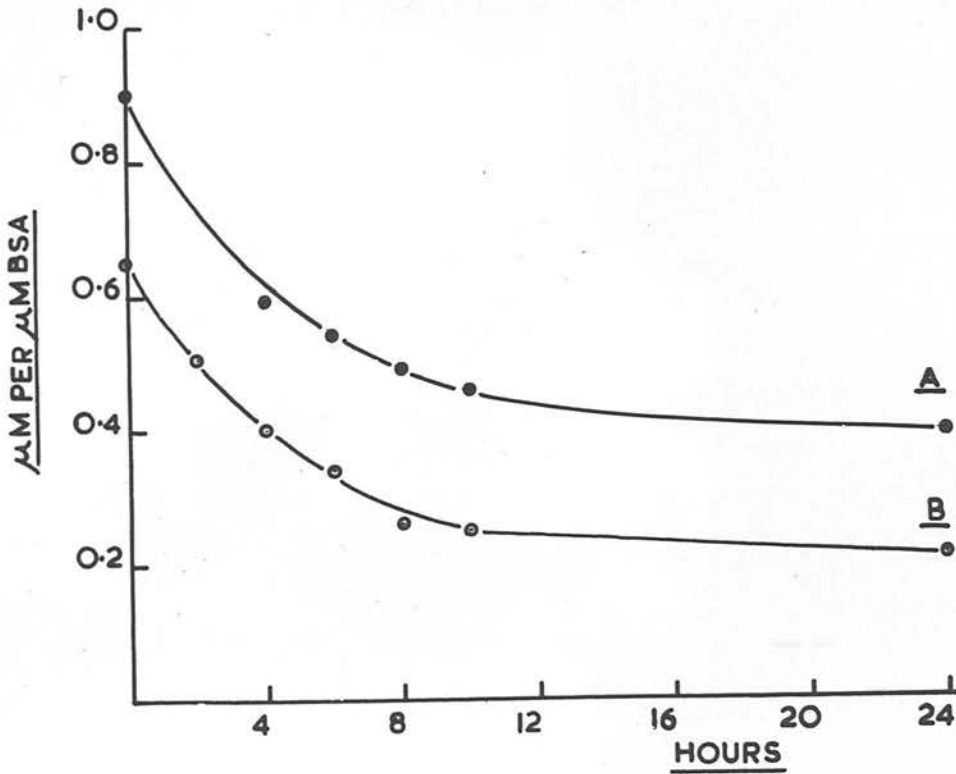
Progress of the BSA-CySSCy Reaction with Time

BSA incubated with CySSCy, in ratio of 1 mole BSA to 0.5 moles CySSCy, at 37°C and pH 7.4. Buffer concentration, 0.2 M Tris/HCl.

Sulphydryl content by pCMB method, CySSCy by the Sullivan and Hess Method.

A. $\frac{1}{2}$ CySSCy concentration in medium which contained 1 μ M BSA.

B. Sulphydryl content of BSA in μ M SH per μ M BSA



group of the BSA was necessary for the reaction with CySSCy. The results also showed that one molecule of bovine mercaptalbumin combined with one $\frac{1}{2}$ CySSCy molecule.

Rate of the Reaction. The previously described experiments have shown that cystine reacted with BSA during an incubation period of 20 hours.

Further studies were conducted to determine the pattern of the reaction within that time and the results of these studies are shown in Table 16 and Figure 6.

From these results it can be seen that the fall in sulphydryl content was paralleled by a decrease in the concentration of CySSCy in the medium.

After ten hours the reaction was virtually complete but it did continue at a slow rate for at least another fourteen hours.

Factors Influencing the Rate of Reaction.

(1) Variations in the Relative Amounts of Reactants. As was to be expected from consideration of the "Law of Mass Action", increase in the amount of cystine relative to the amount of BSA present initially in the reaction mixtures brought about an increase in the rate of the reaction. Table 17 illustrates the effect.

(2) /

(2) Effect of pH. The hydrogen ion concentration of the

reaction medium proved to be extremely critical in determining

the rate of the CySSCy-BSA reaction.

EFFECT ON REACTION RATE OF VARIATIONS
IN REACTANT CONCENTRATIONS

BSA incubated with CySSCy in molar ratios of 4:1, 2:1 and 4:3 at 37°C and pH.7.4

Aliquots taken at intervals up to 6 hours and sulphydryl content determined.

No pH below pH 4.6 was tried because of the danger of

denaturing the protein. (Table 18).

It is generally accepted that disulphides by

| $\mu\text{M CySSCy per}$ $\mu\text{M BSA}$ Initially present | 0 | 0.25 | 0.50 | 0.75 |
|--|------|------|------|------|
| Time (hours) | | | | |
| 0 | 0.68 | 0.68 | 0.67 | 0.67 |
| 2 | 0.67 | 0.66 | 0.61 | 0.49 |
| 3 | 0.67 | 0.61 | 0.51 | 0.39 |
| 4 | 0.70 | 0.55 | 0.46 | 0.32 |
| 5 | 0.64 | 0.57 | 0.42 | 0.26 |
| 6 | 0.67 | 0.55 | 0.36 | 0.23 |

and this oxidation is catalysed by ions of the heavy metals.

Oxidation of the BSA sulphydryl group to albumin

disulphide does not easily occur because of the steric hindrance

making it difficult for a disulphide bond to form between the

(2) Effect of pH. The hydrogen ion concentration of the reaction medium proved to be extremely critical in determining the rate of the CySSCy-BSA reaction.

The reaction rate increased rapidly with increase in the pH above pH 7, yet did not occur to an appreciable extent at acid pH values down to pH 4.6.

No pH below pH 4.6 was tried because of the danger of denaturing the protein. (Table 18).

It is generally accepted that reduction of disulphides by thiols, with the possibility of the formation of mixed disulphides as intermediates, and the disulphide interchange reaction take place through the mercaptide ion. Such reactions would be facilitated by an increase in the pH of the medium, as was found here.

It appears highly probable that the reaction between CySSCy and BSA sulphydryl group is mediated through a mercaptide ion.

(3) Possible Catalysts for the Reaction.

(a) Heavy Metal Ions. Many non-protein thiols are readily oxidised to the corresponding disulphide by oxygen and this oxidation is catalysed by ions of the heavy metals.

Oxidation of the BSA sulphydryl group to albumin disulphide does not easily occur because of the steric factors making it difficult for a disulphide bond to form between the two /

TABLE 19.

EFFECT OF HEAVY METAL IONS UPON CySSCy-BSA REACTION

TABLE 18

A. 1 μ M BSA incubated with 1 μ M CySSCy and 0.2 μ M of 'Catalyst' at 37°C and pH 7.4 for 1 hour. (Total volume, containing phosphate buffer is overall concentration of 0.1 M.

EFFECT OF pH UPON REACTION RATE

0.5 μ M BSA incubated with 0.5 μ M CySSCy at 37°C for 2 hours. pH values obtained by addition of 1 m tris-Hcl buffer of appropriate pH to a final concentration of 0.2 m tris.

Sulphydryl Content
(μ M SH/ μ M BSA)

| pH | BSA controls | BSA + CySSCy |
|-----|--------------|--------------|
| 4.6 | 0.66 | 0.65 |
| 5.6 | 0.66 | 0.65 |
| 6.6 | 0.66 | 0.61 |
| 7.4 | 0.66 | 0.51 |
| 8.4 | 0.66 | 0.30 |

| | |
|--------------------------------------|------|
| BSA + CySSCy | 0.45 |
| BSA + CySSCy + 0.2 μ M Cu^{++} | 0.41 |
| BSA + CySSCy + 0.4 μ M Cu^{++} | 0.39 |

TABLE 19.

EFFECT OF HEAVY METAL IONS UPON CySSCy-BSA REACTION

A. 1 μM BSA incubated with 1 μM CySSCy and 0.2 μM of 'Catalyst' at 37°C and pH 7.4 for 1 hour. 8 ml. Total volume, containing phosphate buffer in overall concentration of 0.1 M.

| 'Catalyst' | Sulphydryl Content (μM SH/ μM BSA) | |
|---------------------------------------|--|---------------|
| | BSA. No CySSCy | BSA. + CySSCy |
| 0 | 0.67 | 0.58 |
| 0.2 μM . SeO_2 | 0.66 | 0.59 |
| 0.2 μM . Fe^{+++} | 0.66 | 0.61 |
| 0.2 μM . Co^{+++} | 0.67 | 0.59 |
| 0.2 μM . Cu^{++} | 0.68 | 0.59 |

EFFECT OF CUPRIC IONS

B. 1 μM BSA incubated with 1 μM CySSCy and 0.2 μM or 0.4 μM Cu^{++} for 3 hours at 37°C and pH 7.4 in total volume 8 mls.

| | | SH content μM SH/ μM BSA |
|--------------------|--|---|
| BSA . No CySSCy | BSA alone | 0.65 |
| | BSA + 0.2 μM Cu^{++} | 0.62 |
| | BSA + 0.4 μM Cu^{++} | 0.59 |
| BSA + CySSCy | BSA + CySSCy | 0.45 |
| | BSA + CySSCy + 0.2 μM Cu^{++} | 0.41 |
| | BSA + CySSCy + 0.4 μM Cu^{++} | 0.39 |

two albumin molecules, (Straessle, 1954; Klotz et al., 1958).

However, in the presence of oxygen it was considered possible that heavy metal ions and possibly selenium dioxide, which is very active in catalysing the oxidation of GSH and CySH (Tsen and Tappel, 1958), would catalyse the formation of a mixed disulphide between the BSA sulphhydryl group and a $\frac{1}{2}$ CySSCy residue. (see also p.85).

Addition of 0.2 μ M of the "catalysts" per μ M BSA to the reaction mixtures did not result in an increased rate of removal of the titratable sulphhydryl from the protein. (Table 19A.)

In a further experiment with increased amounts of cupric ions there was again no significant increase in the extent of the reaction between BSA and CySSCy which could be attributed to a catalytic effect of the copper upon the reaction. (Table 19B.)

(b) Non-protein Thiols. Accepting the mediation of mercaptide ions in thiol-disulphide interactions, any procedure which might alter the amount of mercaptide ion present in the reaction medium could probably bring about changes in the rate of the BSA - CySSCy reaction.

However, addition of small amounts of thioglycollate to the reaction medium did not produce an appreciable change in the reaction rate. This is illustrated in Table 20.

Similar investigations with cysteine as the added thiol also /

also failed to show any significant reaction.

TABLE 20.

ADDED THIOL AND THE RATE OF THE CySSCy-BSA REACTION

1 μ M BSA incubated with 0.5 μ M CySSCy and 0.05 μ M thioglycollic, acid at pH 7.4 and 37°C in 0.2 M tris HCl buffer. Sulphydryl content by pCMB method at intervals.

If the concentrations of these thiols were increased there was no appreciable reaction between them and the protein sulphydryl group. (see later).

| <u>Kinetics of the Reaction.</u> | | <u>Sulphydryl Content</u> (μ M SH / μ M BSA) | |
|----------------------------------|--------------|--|--|
| Time (hours) | BSA + CySSCy | BSA + CySSCy + thioglycollate | |
| 0 | 0.65 | 0.67 | |
| 1 | 0.59 | 0.58 | |
| 2 | 0.55 | 0.56 | |
| 3 | 0.48 | 0.46 | |
| 4 | 0.47 | 0.47 | |
| 5 | 0.44 | 0.40 | |

trations of CySSCy.

For each order of reaction the appropriate function of the concentration of BSA and CySSCy, in the reaction medium, was plotted against time.

Thus, for zero order, the required linear plot was obtained.

When the data in Table 20 were plotted as

shown

also failed to show any stimulation of the reaction.

However, it was a possibility that under the conditions of the reaction the added thiols would be quickly oxidised and so be prevented from functioning as catalysts.

If the concentrations of these thiols were increased there was an appreciable reaction between them and the protein sulphydryl group. (see later).

Kinetics of the Reaction.

The reaction between CySSCy and BSA has been shown to involve the sulphydryl group of the BSA (see Table 15) and at low CySSCy : BSA molar ratios the fall in sulphydryl content was equivalent to twice the concentration of CySSCy present, that is equal to the CySSCy concentration expressed as $\frac{1}{2}$ CySSCy (see Fig. 4 and Table 14).

Attempts were made to determine the order of the reaction between BSA and CySSCy, by graphical means, from data obtained from the reaction of BSA with various concentrations of CySSCy.

For each order of reaction the appropriate function of the concentration of BSA and CySSCy, in the reaction medium, was plotted against time.

None, however, gave the required linear plot except when the data in Table 21 was used.(Fig.7).

When /

Fig. 7.

Equimolar amounts of Bovine Mercaptalbumin and
 $\frac{1}{2}$ CySSCy reacted at pH 7.4 and 37°C

Second Order Plot

$\frac{1}{(a-x)}$ versus time

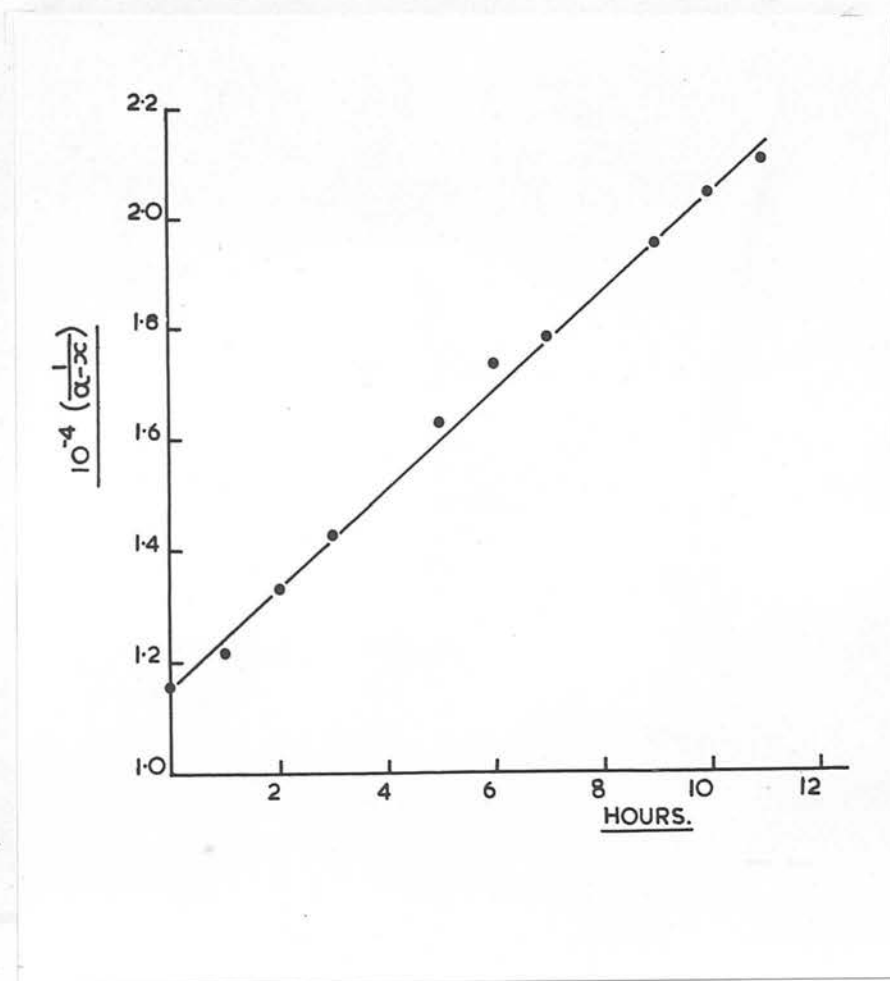


TABLE 21

RATE OF DECREASE IN BSA SULPHYDRYL CONTENT

| Time Hours | Sulphydryl content of BSA | | Sulphydryl lost $M \times 10^{-5} \text{SH/Litre}$ | $\frac{1}{(a - x)} \times 10^{-4}$ |
|---------------|----------------------------------|---|---|------------------------------------|
| | $\mu\text{M SH}/\mu\text{M BSA}$ | $M \times 10^{-5} \text{SH/Litre}$ (a - x) | | |
| 0 | 0.69 | 8.63 | 0 | 1.158 |
| 1 | 0.66 | 8.22 | 0.41 | 1.215 |
| 2 | 0.60 | 7.50 | 1.13 | 1.332 |
| 3 | 0.56 | 7.00 | 1.63 | 1.428 |
| 4 | - | - | - | - |
| 5 | 0.49 | 6.12 | 2.51 | 1.631 |
| 6 | 0.46 | 5.75 | 2.88 | 1.738 |
| 7 | 0.45 | 5.60 | 3.03 | 1.781 |
| 8 | - | - | - | - |
| 9 | 0.41 | 5.12 | 3.51 | 1.952 |
| 10 | 0.39 | 4.89 | 3.74 | 2.045 |
| 11 | 0.38 | 4.75 | 3.88 | 2.110 |

When the initial molar concentrations, considered as bovine mercaptalbumin (which is the sulphhydryl containing portion of BSA) and $\frac{1}{2}$ CySSCy, were equal, the values in Table 21. represented the special case where the second order reaction equation

$$k = \frac{2.303}{t(a-b)} \log \frac{b}{a} \frac{(a-x)}{(b-x)} \quad (I)$$

could be contracted to

$$k = \frac{1}{t} \cdot \frac{x}{a(a-x)} \quad (II)$$

as the initial molar concentrations of the reactants were equal, (Glasstone, 1960).

In the equations (I) and (II) 'k' represents the velocity constant, 'a' and 'b' are the initial concentrations of the reactants and 'x' is the amount of each reacted in a time 't'.

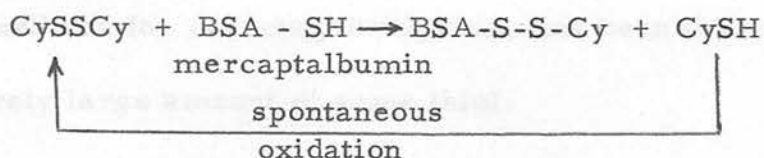
For this special case a plot of $\frac{1}{(a-x)}$ against 't' gave a straight line, of which the slope was equal to 'k', the apparent second order rate constant.

The value obtained for 'k' was 0.247 litres per mole per second.

Substituting in equation (II) the values for the terms 'a', 'a - x' and 'x' from the data in Table 21. the value of 'k' at various /

various time intervals was calculated, and found to equal 0.245 ± 0.014 litres per mole per second.

However, the initial reaction must involve CySSCy and not $\frac{1}{2}$ CySSCy, which does not exist as such; but if the reaction be considered to proceed as follows



the kinetic findings indicating an apparent second order reaction between mercaptalbumin and $\frac{1}{2}$ CySSCy can be explained, providing the oxidation of the CySH to CySSCy is much faster than the reaction of mercaptalbumin with CySSCy.

This is not an unreasonable assumption as CySH has been shown to be oxidised rapidly in alkaline media, (Dixon and Tunnicliffe, 1923).

Reversal of the Reaction.

As the reaction of BSA with CySSCy resulted in a diminution of the sulphydryl content of the protein, and a concomitant loss of cystine from the medium, the formation of a mixed disulphide between the albumin and the non-protein disulphide was postulated.

If this did in fact occur it should have been possible to employ /

employ a reducing agent which would react with the mixed disulphide, liberating protein sulphydryl groups and $\frac{1}{2}$ CySSCy residues.

The results of such a reaction would be detectable by the methods available.

Perhaps one of the most common, and certainly the most specific, methods for reducing disulphides has been the addition of a relatively large amount of some thiol.

This was done here using GSH, in preference to the more easily oxidised CySH, as the added thiol with the results shown in Table 22.

Such treatment produced an increase in the sulphydryl content of the BSA, which had been reacted with CySSCy, and also in the BSA which had not reacted with CySSCy, giving a final value for the sulphydryl content higher than that normally found in BSA. It also appeared to reverse the loss of sulphydryl brought about by reaction with CySSCy.

The extra titratable sulphydryl could possibly have arisen by reduction of the one accessible disulphide bond of BSA, or from reduction of the mixed disulphide which, it has been claimed, (King, 1961), accounts for the fractional sulphydryl content normally found in BSA.

Unfortunately the release of a $\frac{1}{2}$ CySSCy residue on treatment with GSH could not be verified, as measurement of CySSCy /

TABLE 22.

REVERSAL OF CySSCy - BSA REACTION BY GSH

BSA and CySSCy reacted at 37°C and pH 7.4. 10 μ M GSH per μ M BSA added to reaction mixtures and incubated in evacuated Thunberg tubes for 2 hours. Dialysed 20 hours against running water (15-17°C). Sulphydryl content by pCMB method.

| | BSA No CySSCy | | BSA + CySSCy | |
|----------------------------|---------------|----------------|--------------|----------------|
| | No GSH | 10 μ M GSH | No GSH | 10 μ M GSH |
| μ M SH per μ M BSA | 0.64 | 0.91 | 0.25 | 0.90 |

TABLE 23.

PRE TREATMENT OF BSA WITH GSH

BSA incubated "in vacuo" with 10 moles GSH per mole BSA for 2 hours. Dialysed 20 hours against running water. 1 μ M BSA then reacted with 1 μ M CySSCy at pH 7.4 and 37°C. One aliquot of dialysed, GSH-treated BSA kept at 4°C. Sulphydryl content by the pCMB method.

| | BSA. No GSH | | | BSA TREATED WITH GSH | | |
|----------------------------|-------------------------------|------|-----------------|----------------------|----------|-----------------|
| | BSA.no CySSCy 0°C and 37°C | | BSA + CySSCy | BSA 0°C | BSA 37°C | BSA + CySSCy |
| μ M SH per μ M BSA | 0.64 | 0.64 | 0.19 | 0.86 | 0.71 | 0.22 |

CySSCy by the Sullivan and Hess procedure was not possible in the presence of the high GSH concentrations.

Pre-treatment of the BSA with 10 moles GSH per mole BSA, followed by dialysis also led to an increase in the sulphhydryl content of the BSA.

However, incubation of the GSH treated protein at 37°C and pH 7.4 for 20 hours caused a fall in the sulphhydryl content to a value much nearer that normally found. No such fall in sulphhydryl was noticed on incubation of BSA not treated with GSH (Table 23).

The final sulphhydryl content was the same in GSH-treated, and untreated, BSA following reaction with CySSCy.

Having been unable to measure the amount of $\frac{1}{2}$ CySSCy released by GSH treatment attention was turned to another reagent capable of cleaving disulphide bonds, namely cyanide.

The action of cyanide upon BSA was similar to the action of GSH in that it resulted in an increase in the sulphhydryl content of the protein.

Again, on incubation at 37°C for 20 hours, the sulphhydryl content tended to revert to the value normally found and the reversion was more complete in the presence of cupric ions (Table 24). At 4°C there was no fall in the titratable sulphhydryl.

Once /

TABLE 24.

CYANIDE TREATMENT AND BSA SULPHYDRYL CONTENT

BSA solution (1 $\mu\text{M}/\text{ml}$) dialysed 20 hours against 0.25% NaCN solution at 4°C , followed by dialysis against glass distilled water at 4°C . BSA (No cyanide) dialysed against water only.

REACTION OF BSA and CySSCy then carried out at 37°C and pH 7.4 Sulphydryl content by the pCMB method.

| | | Sulphydryl Content ($\mu\text{M SH per } \mu\text{M BSA}$) |
|----------------------------|---|---|
| <u>BSA No cyanide</u> | BSA 4°C | 0.63 |
| | BSA 37°C | 0.63 |
| | BSA + 0.1 $\mu\text{M Cu}^{++}$ | 0.60 |
| | BSA + CySSCy | 0.19 |
| <u>BSA Cyanide treated</u> | | |
| | BSA 4°C | 1.00 |
| | BSA 37°C | 0.71 |
| | BSA + 0.1 $\mu\text{M Cu}^{++}$ (37°C) | 0.64 |
| | BSA + CySSCy | 0.23 |

Once more the final sulphydryl contents, of BSA, pre-treated with cyanide, and BSA not treated in this way, were insignificantly different after both had reacted with CySSCy.

With both GSH and cyanide treatments there was the increase in sulphydryl content above the value of 0.65 sulphydryl groups per molecule which was normally found. This increase tended to disappear on incubation in air at 37°C, especially in the presence of cupric ions suggesting the oxidation of sulphydryl groups more labile, and therefore different, from the sulphydryl group normally present in BSA.

It is probable that the extra sulphydryl arose from the one disulphide group accessible to GSH and cyanide when BSA is in the native state.

Incubation of untreated BSA under these conditions did not lead to a diminution in the titratable sulphydryl content.

After incubation of CySSCy with BSA, dialysis of the reaction mixtures against 0.25% NaCN solution was carried out. Dialysis against glass distilled water at 4°C then removed the excess cyanide. The dialysed solutions were analysed for protein sulphydryl content and protein bound $\frac{1}{2}$ CySSCy residues.

In the estimation of CySSCy one aliquot of each of the solutions, containing 0.5 μ M BSA was treated with sodium cyanide solution, added to a final concentration of 0.9%, for 15 minutes before precipitation of the protein.

This /

This treatment was designed to release any $\frac{1}{2}$ CySSCy residues bound to the protein through disulphide bonds. A measure of the quantity of CySSCy released was obtained by the Sullivan and Hess procedure.

Another aliquot of the solutions treated with cyanide after precipitation of the protein served as a check upon the efficacy of dialysis in removing excess unreacted CySSCy.

BSA-CySSCy reaction mixtures and solutions of BSA alone were treated with cyanide in the same way after dialysis against distilled water instead of cyanide.

It can be seen from Table 25. that no $\frac{1}{2}$ CySSCy remained bound to BSA after dialysis against cyanide and that removal of $\frac{1}{2}$ CySSCy residues was associated with an increase in the sulphydryl content of the protein.

On the other hand, BSA reacted with CySSCy and not dialysed against cyanide, showed a reduced sulphydryl content and was found to be combined with $0.19 \mu\text{M}$ CySSCy per μM BSA by the Sullivan and Hess method. This CySSCy was not removed by dialysis against water.

Care must be exercised in the interpretation of the results obtained from the action of cyanide upon BSA reacted with CySSCy as the action of cyanide, especially in an alkaline medium, as existed here, is complex.

The /

TABLE 25

EFFECT OF CYANIDE UPON BSA SULPHYDRYL CONTENT AND
BOUND $\frac{1}{2}$ CySSCy RESIDUES

| | BSA dialysed against water | BSA + CySSCy | BSA. No CySSCy | BSA. No CySSCy BSA + CySSCy | BSA dialysed against cyanide | CySSCy Standards |
|--|-------------------------------|--------------|----------------|--------------------------------|---------------------------------|---------------------|
| Sulphydryl content (μ M SH per μ M BSA) | 0.64 | 0.20 | 0.82 | 0.82 | 0.82 | - |
| CySSCy content Na CN before BSA precipitation (μ M CySSCy) | 0 | 0.158 | 0 | 0 | 0 | 0.125 |
| Na CN after BSA precipitation (μ M CySSCy) | 0 | 0.063 | 0 | 0 | 0 | 0.116 |
| μ M CySSCy Released from BSA | 0 | 0.095 | 0 | 0 | 0 | - |
| μ M CySSCy Released per μ M BSA | 0 | 0.19 | 0 | 0 | 0 | - |

The reaction of CySSCy with cyanide is



and it is only the cysteine mercaptide ~~ion~~ which is estimated by the Sullivan and Hess method.

The action of cyanide on a mixed disulphide between BSA and $\frac{1}{2}$ CySSCy might favour the formation of the sulphydryl form of BSA rather than CySH or a mixture of the two.

Little is known about the cleavage of such mixed disulphide bonds, (Neubeck and Smythe, 1944), but such an uneven distribution of sulphydryl is suggested by the identical sulphydryl contents of BSA, treated with CySSCy, and untreated BSA after dialysis against cyanide. (Table 25.).

However, the possibilities that the thiocyanate was converted into disulphides by alkali, (Tarvell and Harmish, 1951), or that lanthionine peptides were formed from the protein, do arise.

Lanthionine peptides are more readily attacked by alkali than is CySSCy (Cecil and McPhee, 1959) and can give rise to sulphydryl compounds (Dann et al., 1957).

Thus on account of the apparent complexity of the system no absolute reliability can be placed upon the quantitative results obtained by the Sullivan and Hess procedure but qualitatively the method shows that only in the case where BSA had /

had reacted with CySSCy, and had not been subsequently dialysed against cyanide, was there a positive colour formed with the naphthoquinone sulphonate reagent indicating that $\frac{1}{2}$ CySSCy had been bound to the BSA.

Another point of interest illustrated in Table 25, was the absence of CySSCy from the BSA not reacted with CySSCy but treated with cyanide before precipitation of the protein.

If the fractional sulphydryl content of 0.65 for normal BSA was due to the existence of a mixed disulphide with CySSCy (King, 1961) one would have expected to find an amount of CySSCy present after treatment of the BSA with cyanide.

Unless some other molecule, not detectable by the Sullivan and Hess method was involved in such a mixed disulphide, it would appear that the increase in sulphydryl content found on treatment with GSH or sodium cyanide was due to reduction of a disulphide bond within the BSA molecule.

The latter conclusion was supported by the ease of removal of the extra sulphydryl on incubation at 37°C suggesting the oxidation of a reactive sulphydryl group.

Reaction of BSA with other Thiols TABLE 26.

EFFECT OF VARIOUS THIOLS AND DISULPHIDES
UPON THE SULPHYDRYL CONTENT OF BSA

The finding that BSA reacted rapidly with CySSCy but did not react with CySSCys was confirmed by incubating 1 μ M BSA incubated at 37°C and pH 7.4 in 0.2 M tris buffer with 1 μ M disulphide or 2 μ M thiol for 20 hours in the presence of air. Sulphydryl content by pCMB method. is shown in Table 26.

| Thiol or Disulphide | BSA Sulphydryl Content | |
|----------------------------------|------------------------|------------|
| | μ MSH/ μ M BSA | % Original |
| BSA alone | 0.62 | 100 |
| Cystine | 0.22 | 35.5 |
| Cysteine | 0.23 | 37 |
| Homocystine | 0.30 | 48 |
| Oxidised glutathione | 0.60 | 97 |
| Thioglycollate | 0.48 | 77 |
| 2:3 dimercapto propanol (B.A.L.) | 0.53 | 86 |

Thunberg tube.

In the latter case to ensure as complete a removal of oxygen as possible nitrogen was bubbled through the incubation mixture for 15 minutes before the addition of BSA.

The Thunberg tube was sealed with a cork and the reaction mixture was incubated for 20 hours at 37°C.

Reaction of BSA with other Thiols and Disulphides.

The finding that BSA reacted rapidly with CySSCy but did not react with GSSG prompted an investigation of the reactivities of several thiols and disulphides which were to hand.

The effect upon the sulphydryl content of these compounds is shown in Table 26.

All the thiols and disulphides used, with the exception of GSSG, brought about a significant fall in the sulphydryl content of the BSA.

Cysteine and cystine had the greatest effect and the decrease in the sulphydryl content was the same whether the thiol or the disulphide was used.

This posed the question of whether cysteine needed to be oxidised to cystine before the reaction could take place.

Such an oxidation could have taken place rapidly under the conditions of the reaction illustrated in Table 26.

In an attempt to answer this question BSA was incubated with CySH either in the presence of air or in an evacuated Thunberg tube.

In the latter case to ensure as complete a removal of oxygen as possible nitrogen was bubbled through the incubation mixtures for 5 minutes before the addition of the CySH.

The Thunberg tube was evacuated once the CySH had been added, /

added, filled with nitrogen, shaken for 2-3 minutes and re-evacuated. This procedure was repeated twice more, the tube being finally left evacuated.

The mixtures were then incubated for 20 hours and dialysed against running water (15-17°C) after the reaction to remove unreacted cysteine and cystine.

To evaluate the effect of dialysis on the reaction between CySH and BSA a mixture of the protein and the thiol identical to those incubated was dialysed immediately after mixing of the reactants.

From Table 27 it can be seen that evacuation of the tube reduced the extent of reaction by some 16%.

It would thus appear that the reaction was favoured by the presence of oxygen but the reaction still took place when precautions were taken to exclude air from the reaction.

TABLE 27.

Reaction of CySSCy with Human Plasma.

EFFECT OF AIR ON THE REACTION OF BSA
WITH CySH

| | | Sulphydryl content | |
|--------------------|--------------|---------------------------|---------------|
| | | μ MSH per μ M BSA | % of original |
| In Air | BSA. No CySH | 0.63 | 100 |
| | BSA + CySH | 0.25 | 39.5 |
| In Vacuo | BSA. No CySH | 0.63 | 100 |
| | BSA + CySH | 0.35 | 56 |
| Immediate Dialysis | BSA. No CySH | 0.63 | 100 |
| | BSA + CySH | 0.58 | 92 |

Reaction of cystine with the plasma proteins was found to occur under the same conditions as the reaction with BSA.

(Table 28.)

Treatment of the product of the cystine-plasma reaction with CySH in estimated limiting doses brought about a restoration of the sulphydryl content.

Reaction of CySSCy with Human Plasma.

As a reaction between CySSCy and albumin prepared from bovine plasma had been shown to occur, the possibility of a reaction between the disulphide and freshly drawn plasma was anticipated.

Human plasma from normal persons contains 5.5 - 7.6 g protein per 100 mls, and of this, albumin makes up 3.3 - 5.6 g (Stewart and Dunlop, 1958).

Other proteins, including the sulphydryl containing γ - globulins are also present.

In most cases the oxalated plasma used was pooled on separation from the cells and dialysed overnight at 4°C against a large volume of 0.9% sodium chloride solution.

The protein concentration of the dialysed plasma was determined from spectrophotometric measurements of the optical density of suitably diluted solutions at 260 m μ and 280 m μ according to the procedure of Kalchar, (1947).

Reaction of cystine with the plasma proteins was found to occur under the same conditions as the reaction with BSA. (Table 28.)

Treatment of the product of the cystine-plasma reaction with GSH in evacuated Thunberg tubes brought about a regeneration of the sulphydryl content.

This /

TABLE 28.

PLASMA PROTEIN AND CySSCy EFFECT
ON PROTEIN SULPHYDRYL CONTENT

1 μ M plasma protein incubated with 1 μ M CySSCy at 37°C. pH 7.4 for 20 hours in total volume 8 mls. Buffered by addition of tris-HCl pH 7.4 to 0.2M or without added buffer.
Aliquots treated with 10 μ M GSH per μ M plasma protein in Thunberg tubes for 2 hours at 37°C. Dialysed against running water.
SH content by pCMB method.

| SH Content (μ M SH per μ M protein) | Plasma no CySSCy | | Plasma + CySSCy | | |
|--|------------------|--------------|---------------------|-----------------|--------------------------|
| | Plasma no GSH | Plasma + GSH | Plasma (no tris) | Plasma + CySSCy | Plasma + CySSCy + GSH |
| | 0.50 | 0.68 | 0.49 | 0.24 | 0.68 |
| | | | | | 0.24 |

This was also found in the solutions of plasma proteins not treated with CySSCy. In both cases the sulphhydryl content of the CSH treated material was greater than the untreated protein; as had been found with CSH and cytochrome c and BSA.

TABLE 29

RATE OF THE REACTION OF CySSCy WITH PLASMA, DIALYSED AND UNDIALYSED

One aliquot of pooled plasma dialysed against 0.9% NaCl for 20 hours at 4°C. Another aliquot not dialysed. Plasma protein and CySSCy incubated in equimolar amounts at 37°C, pH 7.4 in 0.2 M Tris-HCl buffer. Aliquots for SH estimation by pCMB method taken at 2 hourly intervals.

| Time (Hours) | Sulphydryl Content (μ M SH per μ M protein) | |
|-----------------|---|-------------------|
| | Dialysed Plasma | Undialysed Plasma |
| 0 | 0.50 | 0.50 |
| 2 | 0.40 | 0.43 |
| 4 | 0.29 | 0.31 |
| 6 | 0.25 | 0.26 |
| 8 | 0.23 | 0.19 |

This was also found in the solutions of plasma proteins not treated with CySSCy. In both cases the sulphydryl content of the GSH treated proteins was greater than the untreated proteins; as had been found with GSH and cyanide treated BSA.

The fall in sulphydryl content of the plasma proteins, assumed to have an average molecular weight of 95,000 was to approximately 40% of the original on treatment with cystine. The same result was obtained, whether Tris buffer had or had not been added, suggesting that the reaction would be possible under physiological conditions.

The value of 0.5 μM sulphydryl per μM plasma protein obtained, assuming the mean molecular weight of 95,000 was equivalent to 72 μM SH per 100 mls of undiluted plasma. This can be compared with the value of 51.3 μM SH per 100mls quoted by Carter (1959).

The possibility that some factor, which might have acted as a catalyst or as an inhibitor, in the reaction with cystine, had been lost during dialysis was investigated. Using plasma, which had been dialysed against 0.9% sodium chloride solution, and plasma, which had not been dialysed, measurements of the rate of reaction with cystine at pH 7.4 and 37°C. were made. (Table 29.)

No significant difference was found between the rate of change /

change in the sulphhydryl content of dialysed plasma and that of undialysed plasma, on reaction with CySSCy.

The Effect of Erythrocytes on Protein reacted with CySSCy.

It is unlikely that the proteins can penetrate the erythrocyte membrane, (Solomon, 1958) which is also impermeable to the physiological disulphides, GSSG and CySSCy, (Eldjarn, et al., 1962).

However, experiments were carried out to see if there existed some mechanism by which the CySSCy, which had reacted with plasma proteins could be released, with a simultaneous regeneration of the protein sulphhydryl groups, on incubation of the plasma with erythrocytes.

Incubation of human erythrocytes with diluted plasma from the same donor unfortunately gave rise to a small amount of haemolysis in most of the experiments, especially when a gas, oxygen or nitrogen, was bubbled through the mixture.

This haemolysis, small in extent though it was, released sufficient haemoglobin sulphhydryl groups, there being six per molecule, to react with an appreciable amount of pCMB rendering interpretation of the measurements of protein sulphhydryl content impossible.

The /

TABLE 30

EFFECT OF ERYTHROCYTES UPON PROTEIN SULPHYDRYL CONTENT

| SH content μ M SH per μ M protein | Plasma no CySSCy | | | | Plasma + CySSCy | | |
|---|--------------------|--------------|---------------------------|--------------------|-----------------|---------------------------|--------------------|
| | No Erythrocytes | Erythrocytes | Erythrocytes + glucose | No Erythrocytes | Erythrocytes | Erythrocytes + glucose | No Erythrocytes |
| | 0.50 | 0.48 | 0.54 | 0.34 | 0.35 | 0.33 | |

The washed cells used in the incubation were prepared from freshly drawn, oxalated blood by separation from the plasma, and washed free of glucose by suspension in 0.9% NaCl at 37°C for 15 minutes. This was repeated twice. The removal of glucose was confirmed by a qualitative glucose oxidase method (Clinistix).

Where cells containing glucose were required only one, rapid washing with 0.9% NaCl was carried out.

On the few occasions when there was no apparent haemolysis at all, the results obtained suggested that erythrocytes had no effect upon the sulphydryl content of the plasma proteins whether they had been reacted with CySSCy or not. (Table 30).

Because of the interference due to released haemoglobin and other sulphydryl containing compounds in the estimation of the sulphydryl content of the plasma proteins the experiments illustrated here were of limited value and the study was not pursued any further.

DISCUSSION

The findings of extensive studies on non-protein thiol-disulphide reactions are being applied directly to reactions involving proteins.

These studies have been carried out with proteins and have demonstrated the formation of mixed disulphides but also the formation of intramolecular disulphides. The following reaction with a disulphide is discussed.

DISCUSSION

Attempts have been made to establish the mechanism of the reactions involving disulphide groups with non-protein disulphides (Hess et al., 1955; 1956; 1957; 1958; 1959).

In the present study the effects of various thiol and disulphides upon the sulphhydryl content of a number of proteins have been studied.

Investigations have been carried out with a variety of proteins and have shown that disulphide groups in proteins are not only formed but can be reformed. The reformation of disulphide groups in proteins is dependent upon the presence of a disulphide donor.

INTRODUCTION

The findings of extensive studies on non-protein thiol-disulphide reactions are not easy to apply directly to reactions involving proteins.

Those studies which have been carried out with proteins have demonstrated the formation of mixed disulphides but many sulphydryl-containing proteins give no evidence of the formation of intermolecular disulphide bonds following reaction with a non-protein disulphide.

Attempts have been made to elucidate the mechanisms of the reactions involving protein sulphydryl groups and non-protein disulphides, (Klotz et al., 1958; Eldjarn and Pihl, 1958b).

In the present work the effects of various thiols and disulphides upon the sulphydryl content of a number of proteins have been studied.

Investigations have been confined to a study of protein sulphydryl groups as these have in general been well characterised and can be estimated easily, while the properties of the disulphide groups within the protein molecules are not so well established.

Factors Influencing the Sulphydryl-Disulphide Interaction.

Of /

Of the five proteins used, namely, bovine serum albumin, human serum albumin, bovine haemoglobin, human haemoglobin and bovine- γ - globulin, not one was shown to react, to any appreciable extent with GSSG to produce a fall in the protein sulphhydryl content.

On the other hand it has been demonstrated that CySSCy brought about a fall in the sulphhydryl content of BSA and bovine haemoglobin, and a similar, though smaller, effect has been obtained with BSA reacted with homocystine.

The ability of CySSCy and homocystine to react while GSSG did not react, reflects the structural differences in the disulphide molecules.

The chemical groupings existing within the disulphide molecules can affect the course of the reaction, which on account of the observed pH dependence is considered to be effected through the mercaptide ion.

As the mercaptide ion is highly nucleophilic any grouping which tends to alter the electron density upon the sulphur atoms of the disulphide bond will tend also to alter the rate at which the mercaptide ion reacts with this bond.

At the pH of the reaction ionisation of some of the groups in the disulphide will occur and the net charge upon the molecule can play an important part in determining the rate of approach of /

of the negatively charged mercaptide ion.

Substitution of the amino groups of cystamine to give NN' diacetyl cystamine was found by Eldjarn and Pihl (1956c) to decrease greatly the rate of reaction with a mercaptide ion.

With GSSG the amino groups of CySSCy are involved in peptide bonds with γ -glutamyl residues. GSSG thus possesses more ionisable carboxyl groups than CySSCy.

Though these considerations probably play some part in determining the possibility of reaction between a disulphide and the protein mercaptide ion, it is most likely that the most important factor in preventing a reaction between GSSG and the proteins is steric hindrance.

GSSG can react readily with small molecular weight thiols such as cysteamine, (Eldjarn and Pihl, 1956c) so that it does appear as if due to the molecular structures, the protein mercaptide ion and the GSSG disulphide bond cannot approach each other closely enough, to permit reaction, and possibly mixed disulphide formation.

The reactivities of the protein sulphydryl groups, reacting as mercaptide ions, depend upon the ionisation constants of the individual sulphydryl groups, which in turn depend upon the presence of electron withdrawing substituents within the molecule /

Various thiols were also found to react with BSA to form a molecule tending to make the ion less nucleophilic.

With bovine haemoglobin it appears that only one sulphhydryl group reacted with CySSCy (p.75) and none with GSSG (p.70) suggesting that steric factors completely excluded GSSG and that one sulphhydryl group was either more accessible to CySSCy or was intrinsically more reactive than the other.

BSA gives only a faint nitroprusside test under normal conditions but reacts readily with iodoacetamide indicating that the sulphhydryl group is 'masked' to some extent from reaction with certain reagents, (Benesch et al., 1955).

This 'masking' of the BSA sulphhydryl group could exclude the large molecule of GSSG but allow CySSCy to react, while homocystine, with a molecular weight a little greater than that of CySSCy, also reacts but at a slower rate.

These reactions which take place between protein sulphhydryl groups and miscellaneous disulphides, so obviously depend upon the accessibility and ionisation of the sulphhydryl groups, that not until something more is known of the amino acid sequence of the proteins and their finer structure will it be possible to get a full picture of the factors affecting the reactions.

Various /

Various thiols were also found to react with BSA to differing extents.

CySH reacted with BSA to the same extent as an equivalent amount of CySSCy, while the reaction of thioglycollate and 2:3 dimercaptopropanol with BSA reduced the protein sulphhydryl content by 23% and 14% respectively. (p.115).

At the pH of the reaction the oxidation of CySH to CySSCy is known to be rapid, (Dixon and Tunnicliffe, 1923) and the differences in reactivity of the non-protein thiols may be due in part to the differences in the ease and the rate of oxidation of the thiols to the corresponding disulphide.

No evidence was obtained from the experiments to suggest that reaction between the thiols and the disulphide bonds of the protein, such as has been found with mercaptoethylamine, (Kolthoff et al., 1958), had occurred.

The Stoichiometry and Mechanism of the CySSCy-BSA reaction.

That the sulphhydryl groups of BSA was involved in the reaction between BSA and CySSCy was shown by the complete lack of reaction following treatment of the BSA with pCMB, one of the most specific reagents for sulphhydryl groups. (p.93).

The treatment of the protein with GSH or cyanide after reaction /

reaction with CySSCy produced a sulphydryl content identical to that of protein reacted with CySSCy and subsequently treated with reagents which split disulphide bonds. (p. 107, p.112).

Only that protein which had been reacted with CySSCy gave a positive result with the Sullivan and Hess procedure after treatment with cyanide. (p.112).

Thus it was established that the BSA sulphydryl group was involved in the reaction and it appeared that $\frac{1}{2}$ CySSCy residues were linked to the BSA through a disulphide bond, cleavable by the cyanide treatment.

The reaction between the sulphydryl containing BSA (BSA - SH) and CySSCy could possibly be expressed by



Such a mechanism involves the formation of a protein disulphide dimer (BSA - SS - BSA).

No direct evidence for or against the formation of such a dimer, resulting from the reaction of CySSCy with BSA was obtained.

However, the amount of $\frac{1}{2}$ CySSCy removed from the medium on reaction with BSA was the same, within the limits of /

of accuracy of the analytical methods, as the loss in protein sulphhydryl. (pp.93,95.)

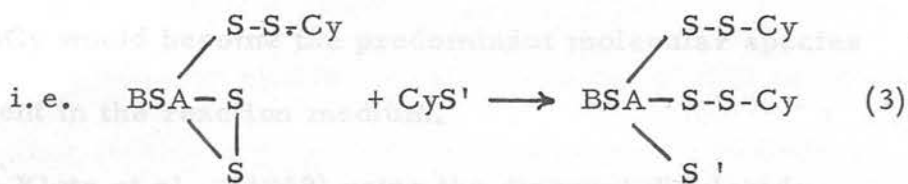
If the protein disulphide dimer had been formed to any appreciable extent it would have released $\frac{1}{2}$ CySSCy residues which, under the conditions of the reaction, would have been reoxidised to CySSCy and so have been included in the estimation of CySSCy. In this case the loss of sulphhydryl content would have been greater than the loss of $\frac{1}{2}$ CySSCy. This was not found.

Klotz et al. (1958) in studies of the reaction of a diphenyl disulphide with BSA considered the formation of a protein disulphide dimer unlikely and found no evidence for it in ultracentrifugal analysis or in molecular weight measurements.

Straessle (1954) has also demonstrated the difficulties associated with albumin dimer formation, prior orientation of the protein molecules being required; although King, (1961) has reported the existence of a dimer in preparations of HSA.

An /

An important possibility arising from equation (I) would be the reduction of intramolecular disulphide bonds by the cysteine mercaptide ion formed.



However, this would lead to an amount of CySSCy, reacting per molecule of BSA of at least twice the value of 0.33 - 0.34 consistently obtained here (pp. 90, 91), and possibly up to 17 times this value if all the disulphide bonds of BSA were involved and not just the most accessible one.

At low CySSCy: BSA ratios the stoichiometry of the reaction shows that 0.33 moles CySSCy were reacting with each mole of BSA which contained 0.65 moles sulphydryl. (pp. 90)

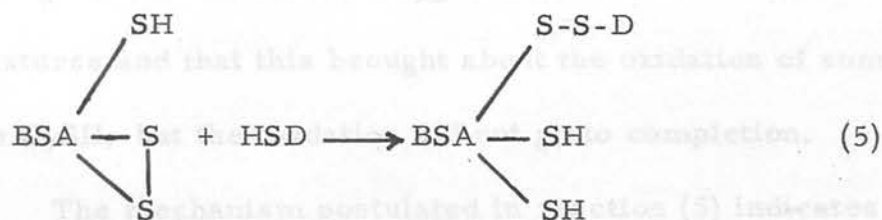
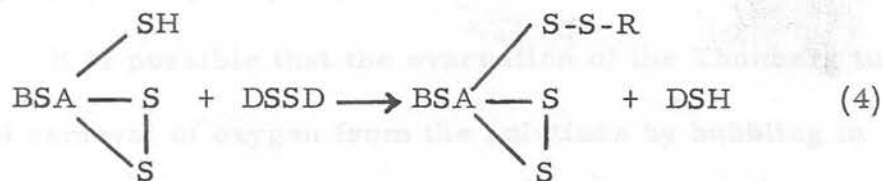
When the CySSCy:BSA ratios were higher the reaction did not go to completion but left approximately 0.2 moles sulphydryl per mole BSA unreacted. (p. 90).

It was possible to remove completely the titratable sulphydryl of the BSA on reaction with CySSCy by reducing the BSA concentration per unit volume by half. When the CySSCy to protein sulphydryl ratio was above pH 4.5 there was /

was no residual sulphhydryl which could be detected by the pCMB method. (p.92).

It is obvious that if no protein disulphide dimer was in the independent of pH over the range 2.5 - 9.2. And the fact formed the mixed disulphide postulated between BSA and CySSCy would become the predominant molecular species present in the reaction medium.

Klotz et al., (1958) using the diphenyl disulphide (DSSD) also obtained consistently a ratio of 0.33 - 0.37 moles DSSD reacting per mole of BSA and suggested that this reaction could be explained by reactions (4) and (5) which did not involve the formation of a protein disulphide dimer.



This scheme of reaction accounted for the observed stoichiometry provided reaction (5) was faster than reaction (4).

Neither oxygen nor heavy metal ions had any effect upon the reaction.

These /

These findings cannot be directly applied to the reaction of BSA with CySSCy as the BSA - DSSD reaction was found to be independent of pH over the range 2.5 - 9.2, and the oxidation to CySSCy of CySH formed in the reaction might very well be much easier than the oxidation of DSH to DSSD.

The lack of any evidence for the presence of CySH in the reaction media (p.92) and the effect which evacuating the Thunberg tubes, in which the CySH - BSA reaction took place, had on lowering the extent of the reaction by 16% (p.117) suggests that oxygen plays a part in the rapid oxidation of any CySH to CySSCy, which then reacts with BSA.

It is possible that the evacuation of the Thunberg tubes and removal of oxygen from the solutions by bubbling in nitrogen, did leave some oxygen dissolved in the reaction mixtures and that this brought about the oxidation of some of the CySH, but the oxidation did not go to completion.

The mechanism postulated in reaction (5) indicates the presence of residual sulphhydryl groups with which the disulphide (DSSD) would not react. They could, however, be detected by titration with silver ions or 4 (p-dimethyl amino benzeneazo) phenyl mercuric acetate.

If the latter mercurial could react with these 'masked' sulphhydryl /

sulphydryl groups left at the conclusion of the reaction it is likely that pCMB would have reacted with them had they been formed in the reaction of CySSCy with BSA.

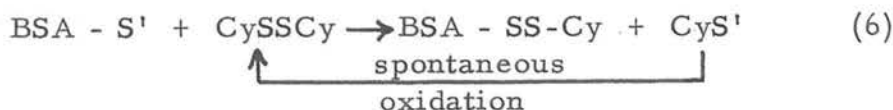
However, it was possible to remove all the protein sulphydryl groups detectable by the pCMB method on reacting CySSCy with BSA.

Treatment of BSA with cyanide or excess GSH did produce an increase in the sulphydryl content detectable by pCMB and this increase in sulphydryl was believed to be the result of the reduction of an intramolecular disulphide bond, a reaction visualised in equation (5).

The extra sulphydryl formed behaved differently from the sulphydryl normally present in the protein on exposure to air at 37°C, especially in the presence of cupric ions; oxidation of the newly formed sulphydryl groups apparently taking place (pp.108, 110).

It thus appears that the mechanism for the reaction between CySSCy and BSA is not identical with that postulated for the reaction between DSSD and BSA.

The stoichiometry of the reaction could also be explained if there was a rapid reoxidation of any CySH formed in the initial reaction of CySSCy with BSA.



In support of this, it was impossible to detect any CySH after incubation of CySSCy with BSA, even though attempts were made to replace the oxygen containing atmosphere with nitrogen.

Incubation of standard CySH solutions under these conditions resulted in a complete loss of CySH, detectable by the pCMB method, suggesting that oxidation had occurred.

Such a representation of the reaction would explain the removal of an amount of $\frac{1}{2}$ CySSCy corresponding to the BSA-sulphydryl lost, no provision for a disulphide dimer being necessary. (p. 95).

In the experiments where CySSCy was present in relatively small amounts, the fall in BSA sulphydryl content suggested that all the CySSCy present had reacted with BSA. (pp. 90, 91.)

Thus the molar sulphydryl content of the protein fell by twice the initial molar concentration of CySSCy.

The oxidation of CySH would have been possible under the conditions of the reaction.

A mechanism similar to that in reaction (6) was suggested by Eldjarn and Pihl, (1956b) to account for their findings in the reaction of cysteamine or cystamine with proteins.

Mixed Disulphides "in vivo"

The reaction of CySSCy with bovine haemoglobin, BSA and plasma under relatively mild conditions of temperature and pH suggests the possibility of an "in vivo" reaction.

Mixed disulphide formation "in vivo" has been demonstrated by Eldjarn and Pihl (1956 a, b) using ^{35}S -labelled cystamine, which on injection into experimental animals combined with plasma proteins, haemoglobin and other sulphydryl containing substances.

Plasma contains, as the free amino acid, 0.8 - 2.0mg CySSCy per 100 ml. (Krebs, 1950) and Stein and Moore, (1954) have shown that the free CySSCy present in freshly shed plasma becomes combined with the proteins during storage.

Eagle et al., (1960) have also shown that $\frac{1}{2}\text{CySSCy}$ residues may be bound to the plasma proteins, while King (1961) postulates the existence of a mixed disulphide containing HSA and CySSCy or GSSG to account for the fractional sulphydryl content normally found for HSA.

However, there is no direct evidence to show that CySSCy is combined with plasma proteins "in vivo".

Although no evidence for any protein-GSSG interaction was found with the proteins used here, there is a wealth of evidence /

evidence indicating that GSSG does in fact react with the sulphhydryl groups of some proteins, (Hopkins and Morgan, 1938; Rapkine, 1938; Hopkins, 1925).

Therefore, if proteins with sulphhydryl groups exist in the tissues in situations where non-protein disulphides may be formed the possibility of mixed disulphide formation must be considered.

The work of Eldjarn and Pihl, (1956a) showed that the "in vivo" formation of mixed disulphides is reversible but whether the reversal is brought about by enzymes or is merely a reflection of a dynamic equilibrium existing between sulphhydryl and disulphide groups in the tissues is not certain.

Enzymes are known which are involved in the reduction of GSSG (Mapson and Goddard, 1951; Conn and Vennesland 1951), and CySSCy (Romano and Nickerson, 1954), having as their coenzymes NADPH₂ and NADH₂ respectively, while an enzyme which catalyses a transhydrogenation between GSH and homocystine in liver, has been reported by Racker, (1954).

More recently enzymes which bring about the reduction of protein disulphide bonds have been shown to exist in yeast, (Nickerson and Falcone, 1956), and in peas and other plants, (Hatch and Turner, 1960).

Glutathione /

Glutathione in the presence of glutathione reductase and a system capable of producing NADPH_2 has been found to reduce CySSCy, homocystine, cystamine and N-alkyl derivatives of cystamine, (Pihl, Eldjarn and Bremer, 1957) while the reduction of those disulphides capable of penetrating the erythrocyte membrane has been shown to occur in erythrocytes, (Eldjarn, Bremer and Borresen, 1962).

A reasonable assumption is that a dynamic equilibrium exists "in vivo" between sulphydryl and disulphide groups and, if the reaction rates are high enough, any variation in the ratio of total disulphide to total sulphydryl, brought about by oxidation or reduction of any one of the molecular species, will presumably affect the concentration of all the other components.

It follows that appreciable amounts of mixed disulphides may exist in tissues and that their biochemical properties, of which so little is yet known, may well be of importance in regulating the influence of sulphydryl compounds in cell metabolism.

Thus a mechanism of biochemical control is suggested involving the conversion of sulphydryl groups to disulphide, including mixed disulphide, groups as part of a specific, reversable inhibition of sulphydryl-containing enzymes.

Possibly /

Possibly by this means the activities of the enzymes may be regulated; mixed disulphides playing an important part, as their formation in many cases would probably, from steric considerations, be easier than the formation of protein disulphide dimers.

As the main role of GSH appears to be the prevention of oxidation of sulphydryl groups, (Barron, 1951; Racker, 1955) the rate at which enzymes could be inhibited or reactivated would depend upon the amount of GSH within the cells.

The level of GSH in the cells and the tissues may be influenced by thyroxine, vitamin B₁₂, growth hormone and the adrenal hormones, (Jocelyn, 1959).

These factors may be involved in adjusting the GSH level to meet the requirements of the tissues.

It follows that if there is a deficiency of any component in the mechanism for reduction of the disulphides the functions of the cell or tissue may be impaired.

The haemolysis, which occurs in humans with the genetically-determined erythrocyte glucose-6-phosphate dehydrogenase deficiency, appears to be an example of the result of such an impairment, (Carson, 1960), sulphydryl groups having repeatedly been postulated to be of importance for /

for the viability of erythrocytes, (Fegler, 1952).

A system therefore seems possible, involving mixed disulphides, which could conceivably play some part in the regulation of the metabolism of cells and tissues, with GSH and glutathione reductase occupying an important central position.

Inhibition of Sulphydryl-Enzymes by Disulphides.

The inhibition of enzymic activity by GSSG or CySSCy has been regarded as one of the criteria for determining the existence of essential sulphydryl groups in the enzymes, (Barron, 1951; Hopkins and Morgan, 1938; Mirsky and Anson, 1935).

The present work suggests that care should be exercised in drawing conclusions regarding the presence or absence of an essential sulphydryl group in an enzyme, if GSSG is used.

Obviously, if an enzyme were to exist, which required sulphydryl groups, but these were "masked" to some extent the reaction with GSSG might be prevented by steric hindrance. Erroneous conclusions could thus be drawn.

CySSCy should also be used as a potential inhibitor where doubts exist.

SUMMARY

1. A method has been developed for the estimation of protein sulphhydryl content. This method was used in a study of the interactions of a few biologically important sulphhydryl containing proteins with non-protein thiols and disulphides.
2. No interaction was detected between C550 or C5H and the proteins, namely, bovine and human haemoglobin, bovine γ -globulin and bovine and human serum albumins, under the conditions employed.

High concentrations of C5H brought about the precipitation of the bovine γ -globulin.

SUMMARY

3. C550 reacted with bovine haemoglobin, removing one sulphhydryl group per molecule, and with bovine serum albumin. The latter reaction was studied in detail.
4. Equimolar amounts of C550 and BSA reacted at 37°C and pH 7.4. The reaction involved 75% of the available protein sulphhydryl content. Decrease in the C550₀ - absorbance at 240 m μ was 1.5 times that brought about by the reaction of C550 with BSA.

SUMMARY

1. A method has been developed for the estimation of protein sulphydryl content. This method was used in a study of the interactions of a few biologically important sulphydryl containing proteins with non-protein thiols and disulphides.
2. No interaction was detected between GSSG or GSH and the proteins, namely, bovine and human haemoglobins, bovine γ - globulin and bovine and human serum albumins, under the conditions employed.

High concentrations of GSH brought about the precipitation of the haemoglobins.

3. CySSCy reacted with bovine haemoglobin, removing one sulphydryl group per molecule, and with bovine serum albumin. The latter reaction was studied in detail.
4. Equimolar amounts of CySSCy and BSA reacted at 37°C and pH 7.4. This reaction removed 75% of the titratable protein sulphydryl content. Increase in the CySSCy - mercaptalbumin molar ratio to 4.5:1 brought the protein sulphydryl content to zero.

The /

The extent of the removal of CySSCy or protein sulphydryl indicated that one molecule of CySSCy was reacting with two molecules mercaptalbumin.

5. The reaction requires an intact protein sulphydryl group. Blocking of the sulphydryl group with p-chloro-mercuribenzoate completely abolished the reaction.

The pH dependence of the reaction suggests that it is mediated through the mercaptide ion.

Addition of catalytic amounts of heavy metal ions or non-protein thiols had no effect upon the reaction.

6. Reaction rate studies indicated second order kinetics for the reaction between mercaptalbumin and $\frac{1}{2}$ CySSCy, a finding explicable by postulating a rapid spontaneous oxidation to CySSCy of any CySH produced in the reaction,

7. Regeneration of the BSA sulphydryl group, removed by reaction with CySSCy, was brought about by reagents (GSH and Cyanide) which react with disulphide bonds. Some of the sulphydryl produced appeared to arise from the reduction of intramolecular disulphide bonds.

8. /

8. The findings enumerated above are compatible with the formation of a mixed disulphide between mercaptalbumin and $\frac{1}{2}$ CySSCy residues. No evidence for the formation of a BSA disulphide dimer was obtained.

9. BSA was found to react with other non-protein thiols and disulphides but not as readily as with CySSCy or CySH.

CySSCy also reacted with human plasma under mild conditions of temperature and pH, causing a decrease in the protein sulphydryl content.

10. The factors which affect thiol-disulphide interactions and the possible role of mixed disulphides are discussed.

ACKNOWLEDGMENTS

The work reported in this thesis was carried out in the Department of Clinical Chemistry, Royal Infirmary, Edinburgh, under the guidance of Dr. P.C. Jocelyn, to whom I am grateful for his constructive suggestions and helpful advice.

I am also indebted to Dr. C.P. Stewart for the advice and encouragement he has given.

My thanks are also due to the Scottish Hospital Endowments Research Trust for the full time grant by means of which this research was undertaken.

REFERENCES

- Allen, D.W., and Jandl, J.H., (1961) J. Clin. Investigation, 40, 454.
- Allison, A.C. and Cecil, R. (1958) Biochem. J., 69, 27.
- Arnold, V. (1910) Z. Physiol. Chem., 70, 300.
- Balls, A.K., and Lineweaver, H. (1939a) J. Biol. Chem., 130, 669.
- Balls, A.K., and Lineweaver, H. (1939b) Nature, London, 144, 513.
- Barron, E.S.G. (1951) Advances in Enzymology, 11, 201.
- Benesch, R. and Benesch, R.E. (1948) Arch. Biochem., 19, 35.
- Benesch, R.E., Lardy, H.A., and Benesch, R. (1955) J. Biol. Chem., 219, 663.
- Benesch, R., and Benesch, R.E. (1956) Fed. Proc., 15, 218.
- Bersin, T., and Steudel, J. (1938) Chem. Ber., 71, 1015.
- Bhattacharya, S.K., Robson, J.S., and Stewart, C.P. (1955) Biochem. J., 60, 696.
- Boyer, P.D. (1954) J. Amer. Chem. Soc., 76, 4331.
- Boyer, P.D. (1960) The Enzymes. Vol. 1, p.511, Academic Press, New York.
- Ed. Boyer, P.D. et al. (1960) Biochem. J., 52, 192.
- Carson, P.E. (1960) Fed. Proc., 19, 995.
- Carter, J.R. (1959) J. Biol. Chem., 234, 1705.
- Cecil, R., and McPhee, J.R. (1959) Advances in Protein Chemistry, 14, 323.
- Cecil, R., and Snow, N.S. (1962) Biochem. J., 82, 255.
- Clarke, /

- Clarke, H.T. (1932) J. Biol. Chem., 97, 235.
- Cohn, E.J., Strong, L.E., Hughes, W.L., Mulford, D.J.,
Ashworth, J.N., Melin, M., and Taylor, H.L. (1946)
J. Amer. Chem. Soc., 68, 459.
- Cohn, E.J., Hughes, W.L., and Weare, J.H. (1947)
J. Amer. Chem. Soc., 69, 1753.
- Conn, E.E., and Vennesland, B. (1951) J. Biol. Chem.,
192, 17.
- Dann, J.R., Oliver, G.L., and Gates, J.W. (1957)
J. Amer. Chem. Soc., 79, 1644.
- Dixon, M., and Tunnicliffe, H.E., (1923) Proc. Roy. Soc.,
B 94, 266.
- Dohan, J.S., and Woodward, G.E. (1939) J. Biol. Chem.,
129, 393.
- Drabkin, D.L. (1949) Arch. Biochem., 21, 224.
- Eagle, H., Ozama, V.I., and Piez, K. (1960)
J. Biol., Chem., 235, 1719.
- Eldjarn, L., and Nygaard, O. (1954) Arch. Internat. de
Physiol., 62, Fasc., 4, 476.
- Eldjarn, L., and Pihl, A. (1956a) J. Biol., Chem., 223, 341.
- Eldjarn, L. and Pihl, A. (1956b) Proc. Fourth International
Conference on Radiobiology. p.249
Ed. Mitchell, J.S. et al., London.
- Eldjarn, L., and Pihl, A. (1956c) J. Biol. Chem., 225, 499.
- Eldjarn, L., Bremer, J., and Borresen, H.C. (1962)
Biochem. J., 82, 192
- Fegler, G. (1952) Nature, London. 170, 624.
- Franklin, E.C., Kinkel, E.G., Muller-Eberhard, H.J., and
Holman, H.R. (1957) Ann. Rheum. Dis.,
16, 315.

French, /

- French, D., and Edsall, J.T. (1945) *Advances in Protein Chemistry*, 2, 278.
- Frensdorff, H.K. (1953) *J. Amer. Chem. Soc.*, 75, 5167.
- Glasstone, S. (1960) *Textbook of Physical Chemistry*, p.1055, MacMillan, London.
- Gutter, F.J., Sober, H.A., and Peterson, E.A. (1956) *Arch. Biochem. Biophys.*, 62, 427.
- Harris, L.J. (1923) *Proc. Roy. Soc. London. B* 94, 426.
- Hatch, J.D., and Turner, J.F. (1960) *Biochem. J.*, 76, 556.
- Heffter, A. (1907) *Mediz. Naturwiss. Arch.*, 1, 81.
- Hellerman, L. (1943) *J. Biol. Chem.*, 147, 443.
- Herriott, R.M. (1947) *Advances in Protein Chemistry*, 3, 169.
- Hopkins, F.G. (1921) *Biochem. J.*, 15, 286.
- Hopkins, F.G. (1925) *Biochem. J.*, 19, 787.
- Hopkins, F.G., and Morgan, E.J. (1938) *Biochem.*, 32, 611.
- Huggins, C., Tapley, D.F., and Jensen, E.V. (1951) *Nature, London*, 167, 592.
- Hughes, W.L. (1947) *J. Amer. Chem. Soc.*, 69, 1836.
- Hughes, W.L. (1949) *Gold Spring Harbour Symposium Quant. Biol.*, 14, 79.
- Hughes, W.L. and Straessle, R. (1950) *J. Amer. Chem. Soc.*, 72, 454.
- Hughes, W.L. (1954) In "The Proteins" Vol. II B p.682 Ed. Neurath, H. and Bailey, K. Academic Press, New York.
- Ingram, V.M. (1955) *Biochem. J.*, 59, 653.
- Ingram, V.M. (1957) *Biochem. J.*, 65, 760.
- Jocelyn /

- Jocelyn, P.C. (1959) Symp. Biochem., Soc., 17, 43.
- Jocelyn, P.C. (1960) Biochem. J., 77, 363.
- Kalchar, H.M. (1947) J. Biol. Chem., 167, 462.
- King, T.P. (1961) J. Biol. Chem., 236, pC 5.
- Klebanoff, S.J. (1957) Biochem. J., 65, 423.
- Klotz, I.M., Urquhart, J.M., Klotz, T.A. and Ayers, J.
(1955) J. Amer. Chem. Soc., 77, 1919.
- Klotz, I.M. and Ayers, J. (1957) J. Amer. Chem. Soc.
79, 4078.
- Klotz, I.M., Ayers, J., Ho, J.Y.C., Horowitz, M.G., and
Heiney, R.E. (1958)
J. Amer. Chem. Soc., 80, 2132.
- Kolthoff, I.M., and Harris, W.E.; (1945) Ind. Eng. Chem.
Anal. Ed., 18, 161.
- Kolthoff, I.M. and Stricks, W. (1950) J. Amer. Chem. Soc.,
72, 1952.
- Kolthoff, I.M., Stricks, W., and Kapoor, R.C. (1955)
J. Amer. Chem. Soc., 77, 4733.
- Kolthoff, I.M., Anastasi, A., Stricks, W., Tan, B.H., and
Deshmukh, G.S. (1957)
J. Amer. Chem. Soc., 79, 5102.
- Kolthoff, I.M., Anastasi, A., and Tan, B.H. (1958)
J. Amer. Chem. Soc., 80, 3235.
- Krebs, H.A., (1950) Ann. Rev. Biochem., 9, 417.
- Lamfrom, H., and Nielsen, S.O. (1958) Compt. Rend. Trav.
Lav. Carlsberg. Ser. Chem., 30, 349.
- Lecher, H. (1920) Chem. Ber., 53, 591.
- Linderstrom-Lang, K., and Jacobsen, C.F., (1941)
J. Biol. Chem., 137, 443.
- Livermore, A.H., and Muecke, E.C. (1954)
Nature, London, 173, 265.

- Racker, E. (1951) 'Glutathione', A Symposium p.155.
- Mapson, L. W., and Goddard, D. R., (1951) Biochem. J., 49, 592.
- Mapson, L. W. (1953) Biochem. J., 55, 714.
- Markus, G., and Karush, F. (1957) J. Amer. Chem. Soc., 79, 134.
- Martin, H. and McIlwain, H. (1959) Biochem. J., 71, 275.
- Mazia, D. (1954) In 'Glutathione' A Symposium. p.209.
Ed. Colowick, S.P. et al.,
Academic Press, New York.
- McCormack, S., Goldzieher, J. W., and Besch, P.K. (1960) Biochem. Biophys. Acta., 38, 293.
- McIlwain, H. (1959) Biochem. Soc. Symposium. 17, 76.
- Mirsky, A. E., and Anson, M. L. (1935) J. Gen. Physiol., 18, 307.
- Mirsky, A. E., and Pauling, L. (1936) Proc. Nat. Acad. Sci. U.S. 22, 439.
- Morner, K. A. H. (1899) Z. Physiol. Chem., 28, 595.
- Murayama, M. (1958) J. Biol. Chem., 233, 594.
- Neubeck, C. E., and Smythe, C. V., (1944) Arch. Biochem., 4, 435.
- Nickerson, W. J. and Falcone, G. (1956) Science, 124, 722.
- Patterson, J. W., and Lazarow, A. (1955) in 'Methods of Biochemical Analysis', Vol. 2 p.259.
Ed. Glick, D. Interscience Publishers,
New York.
- Perlzweig, W. (1932) Science, 76, 435.
- Pihl, A., and Eldjarn, L. (1957) 'Advances in Radiobiology' p.152. Ed. De Henessy, et al.,
Oliver & Boyd, Edinburgh.
- Pihl, A., Eldjarn, L., and Bremer, J. (1957) J. Biol. Chem., 227, 339.

- Racker, E. (1954) 'Glutathione, A Symposium' p.165,
Ed. Colowick, S.P. et al., Academic
Press, New York.
- Racker, E. (1955) J. Biol. Chem., 217, 867.
- Rapkins, L. (1938) Biochem. J., 32, 1729.
- de Rey-Pailhade, J. (1888) Compt. Rend. Acad., 106, 1683.
- Riggs, A.F. (1953) J. Gen. Physiol., 36, 1.
- Riggs, A.F., and Wolbach, R.A. (1956) J. Gen. Physiol.,
39, 585.
- Romano, A.H., and Nickerson, W.S. (1954) J. Biol. Chem.,
208, 409.
- Schelling, V. (1932) J. Biol. Chem., 96, 17.
- Segal, H.L., and Boyer, P.D. (1953) J. Biol. Chem.,
204, 265.
- Shifrin, S., and Kaplan, K.O. (1960) 'Advances in Enzymology'
22, 337.
- Solomon, A.K. (1958) Ann. N.Y. Acad. Sci., 75, 180.
- Stein, W.H., Kunkel, H.G., Cole, R.D., Spackman, D.H.,
and Moore, S. (1957)
Biochem. Biophys. Acta., 24, 640.
- Stein, W.H., and Moore, S. (1954) J. Biol. Chem., 211, 915.
- Stewart, C.P. and Dunlop, D.M. (1958) 'Clinical Chemistry
in Practical Medicine, p.326.
E. & S. Livingstone, Edinburgh.
- Straessle, R. (1954) J. Amer. Chem. Soc., 76, 3138.
- Stadtman, E.R. (1953) J. Biol. Chem., 203, 47.
- Sullivan, M.X., and Hess, W.C. (1937) J. Biol. Chem.,
117, 425.
- Swenson, A.D., and Boyer, P.D. (1957) J. Amer. Chem.
Soc., 79, 2174.
- Tabachnick, /

- Tabachnick, M., Eisen, H.N., and Levine, B. (1954)
Nature, London, 174, 701.
- Tarvell, D.S. and Harnish, D.P. (1951) Chem. Reviews,
49, 1.
- Thomson, C.G., and Martin, H. (1959) Symp. Biochem.
Soc., 17, 21.
- Tsen, C.C., and Tappel, A.L. (1958) J. Biol. Chem.,
233, 1230.
- Wikberg, E., (1953) Nature, London, 172, 398.
- Woodward, G.E., and Fry, E.G. (1932) J. Biol. Chem.,
97, 465.